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**RETHINKING MECHANISMS OF ACTIN PEDESTAL FORMATION BY
ENTEROPATHOGENIC *ESCHERICHIA COLI* IN THE CONTEXT OF
MULTIPLE SIGNALING CASCADES**

A Dissertation Presented

By

PAMELA JOYCE SAVAGE

Submitted to the Faculty of the

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Graduate School of Biomedical Sciences

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 20, 2007

Molecular Genetics and Microbiology

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) is one of many bacterial and viral pathogens that can exploit the eukaryotic actin cytoskeleton for its own purposes. EPEC injects its own receptor, Tir, into the host cell plasma membrane where, upon binding the bacterial adhesin, intimin, can trigger actin assembly beneath bound bacteria resulting in characteristic actin “pedestals”. The formation of these lesions is thought to be critical for bacterial colonization, and can also provide insight into actin dynamics of mammalian cells. EPEC Tir stimulates multiple signaling pathways converging on a central actin nucleation promoting factor, N-WASP. The best-characterized pathway of actin pedestal formation also involves the eukaryotic adaptor protein, Nck, but at least two Nck-independent signaling cascades have also been identified. Multiple aspects of Tir-mediated signaling cascades remain unclear. For example, although Nck can directly bind and activate N-WASP, current models of Tir-mediated, Nck-dependent actin signaling postulate an indirect interaction between Nck and N-WASP mediated by one or more unidentified host factors. Additionally, the relationship of this pathway to the Nck-independent pathways is unknown. To better understand Tir-mediated actin assembly, a detailed and quantitative analysis of the domain requirements of Nck and N-WASP for pedestal formation was conducted. The results indicate that, contrary to previously favored models, Nck is unlikely to require additional host factors to bind N-WASP during pedestal formation, but apparently directly stimulates this nucleation promoting factor. In addition, the results show that the Nck-dependent and -independent pathways target distinct regulatory domains of N-WASP.

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CHAPTER I

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC), the causative agent of infantile diarrheal illness in developing countries, belongs to a family of human enteric pathogens that, upon colonization of the intestinal epithelium, promote the generation of hallmark 'attaching and effacing' lesions (Nataro and Kaper, 1998). Included in this family of bacteria are the notable human pathogen enterohemorrhagic *E. coli* O157:H7 (EHEC), the rabbit enteropathogenic *E. coli* serotype O103:H2 (RPEC) and the mouse pathogen *Citrobacter rodentium*, all of which can colonize the intestine of their respective hosts and produce A/E lesions. These structures are distinguished by the localized destruction of the intestinal microvilli, the intimate attachment of the bacterium with the host cell and the accumulation of filamentous (F)- actin beneath the sites of bacterial adhesion (Donnenberg and Whittam, 2001; Kaper *et al.*, 2004; Knutton *et al.*, 1989). Though the purpose of these pedestals still remains unknown, disruption of genes critical for the formation of these structures has a consequence for bacterial colonization. In rabbit and mouse infection models, using RPEC and *C. rodentium*, respectively, mutants lacking two critical bacterial factors, intimin and Tir (translocated intimin receptor) were unable to generate A/E lesions or promote clinical disease (Deng *et al.*, 2003; Marches *et al.*, 2000).

Within the bacterial chromosome lies a contiguous 35kb pathogenicity island known as the locus of enterocyte effacement (LEE) encoding all of the genes necessary for EPEC-mediated actin assembly (Elliott *et al.*, 1998; Jerse *et al.*, 1990; McDaniel and Kaper, 1997). Included on the LEE are the components for a bacterial type III secretion system responsible for delivering a plethora of bacterial effectors into the host cell (Jarvis *et al.*, 1995). An increasing number of effectors, located both on the LEE and in other loci, have been identified, including EspA, EspB, EspD, EspF, EspG, CifA, Map, and Tir (EspE) (Abe *et al.*, 1998; Elliott *et al.*, 1998; Elliott *et al.*, 2001; Kenny *et al.*, 1996; Kenny *et al.*, 1997; Kenny and Jepson, 2000; Marches *et al.*, 2003; McNamara *et al.*, 2001; Wolff *et al.*, 1998). Spanning the inner and outer membranes of the bacterium is a needle complex comprised several bacterial proteins, including EscC, EscJ, EscN and EscF (Daniell *et al.*, 2001; Wilson *et al.*, 2001), which connects to a filament composed of EspA (Knutton *et al.*, 1998), in whole generating a conduit through which effectors travel, ultimately arriving in the host cell cytoplasm through a pore in the plasma membrane generated by the type III translocators EspB and EspD (Ide *et al.*, 2001; Ogino *et al.*, 2006; Sekiya *et al.*, 2001).

The most well-defined type III effector, Tir, is vital for EPEC-mediated actin assembly. Initially identified as a type III secreted effector in 1997 by Kenny *et al.*, Tir serves as a receptor for the bacterial outer membrane protein, intimin. Deletion of the genes encoding either Tir or intimin abolishes actin accumulation beneath the bound bacteria and abolishes the bacterium's ability to colonize the host (Deng *et al.*, 2003; Kenny *et al.*, 1997). When Tir is ectopically expressed in cultured mammalian cells and clustered in the membrane, F-actin accumulates at Tir foci, indicating that this protein is

the only type III secreted effector required for EPEC pedestal formation (Campellone *et al.*, 2004).

To generate actin pedestals, Tir must be translocated through the type III secretion system into the mammalian cell, bind to intimin on the bacterial surface, and stimulate actin assembly. Though no canonical translocation signal has been identified, the Tir N-terminal region mediates its translocation, because deletion of this region completely impairs Tir secretion (Crawford and Kaper, 2002). Upon delivery into the host cell, Tir is inserted into the plasma membrane in a hairpin-like conformation with the N- and C-terminal regions of the protein located in the host cytoplasm and the central region of the protein exposed in the extracellular milieu (de Grado *et al.*, 1999). The Tir extracellular domain is then recognized by intimin, a step that results in the intimate attachment that is required for EPEC colonization. Tir-intimin binding also results in clustering of Tir in the plasma membrane, an essential step in triggering actin assembly (Campellone *et al.*, 2004). Indeed, artificial clustering of Tir using an antibody against the extracellular portion of the effector promotes actin condensation, and clustering of the cytoplasmic domain of Tir in the absence of any Tir extracellular domain results in localized actin assembly (P. Radharkrishnan, thesis). Finally, the intracellular domains of Tir mediate the last step of actin pedestal formation, the recruitment of the actin machinery to the sites of bacterial attachment. The N-terminus has been shown to interact with actin-binding proteins such as α -actinin, and may play a role in pedestal formation (Goosney *et al.*, 2000). However, clustering of the C-terminal domain, even in the absence of the N-terminus of Tir, is sufficient to trigger actin assembly (Campellone *et al.*, 2004).

N-WASP and Arp2/3 are required for EPEC pedestal formation.

The ability of EPEC Tir to generate pedestals is due to its capacity to stimulate actin assembly via the N-WASP-Arp2/3 pathway, one of the best-defined pathways to actin filament formation. Arp2/3 is a seven-member complex that under basal conditions has little ability to stimulate the nucleation of G-actin. However, in the presence of a nucleation promoting factor, such as N-WASP (neuronal Wiskott Aldrich syndrome protein), Arp2/3 nucleates monomeric or globular (G-) actin into filamentous (F-) actin, generating filaments characteristically branched at 70° angles (Goley and Welch, 2006). Both N-WASP and the Arp2/3 complex accumulate at the tips of EPEC-induced actin pedestals (Goosney *et al.*, 2001; Kalman *et al.*, 1999) and EPEC does not form pedestals on cells that are deficient in N-WASP or that overexpress dominant-negative forms of N-WASP that inhibit Arp2/3 function (Kalman *et al.*, 1999).

The nucleation promoting factor N-WASP is under tight regulator control, and acts as an “integrator” of multiple stimuli to regulate actin nucleation by Arp2/3. As such, N-WASP is comprised of four major domains, each with a different function (Figure 1). The C-terminal verprolin-connector-acidic (VCA) domain is the “effector” domain, as the V subdomain binds actin monomers while the CA subdomain interacts with the Arp2/3 complex, stimulating the nucleation of G-actin to F-actin. The N-terminus of N-WASP consists of the WASP homology 1 (WH1) domain, the GTPase binding domain (GBD), and the proline-rich domain (PRD), each of which contributes to the activity of N-WASP. When N-WASP is in the basal, or autoinhibited, state, the VCA domain is sequestered by an interaction between the C subdomain and the GBD, and

activation signals disrupt the N-WASP autoinhibited state, freeing the VCA domain to interact with the Arp2/3 complex (Figure 1; Takenawa and Suetsugu, 2007).

Activation of N-WASP can occur by through its interaction with phospholipids, small GTPases or SH3 domain-containing proteins, and each of the N-terminal N-WASP domains is capable of receiving input from different sources (Prehoda *et al.*, 2000; Rohatgi *et al.*, 2000; Rohatgi *et al.*, 2001). The WH1 domain binds to a proline-rich region of members of the WASP-interacting protein (WIP) family. Included in this group of proteins are WIP, the WIP-like proteins WICH/WIRE and the neuronally expressed CR-16 (Aspenstrom, 2002, 2004; Ho *et al.*, 2001; Kato *et al.*, 2002; Ramesh *et al.*, 1997). Interestingly, WIP can function as both a positive and negative regulator of N-WASP. Its inhibitory activity can be abrogated by Toca-1, which acts in concert with Cdc42 to stimulate N-WASP function (Ho *et al.*, 2004). In contrast, N-WASP-WIP interaction appears to play an activating function during actin assembly promoted by vaccinia virus (Moreau *et al.*, 2000). A small basic region located in the N-WASP GBD binds to phosphoinositides, whereas the CRIB (Cdc42-Rac interactive binding) sequence within the GBD binds small GTPases, like Cdc42, which is an important for the generation of actin-rich structures such as filopodia (Miki *et al.*, 1998; Rudolph *et al.*, 1998). While Cdc42 and phosphoinositides can individually activate N-WASP, together they act synergistically (Rohatgi *et al.*, 1999; Torres and Rosen, 2006). SH3- and SH2-containing adaptors, such as Nck, Grb2 and CrkII, can utilize their SH3 domains to bind and activate N-WASP via its PRD, while their SH2 domains serve as adaptors to link activation of the actin machinery to tyrosine phosphorylation, often located at the plasma membrane (Benesch *et al.*, 2002; Carrier *et al.*, 2000; Rohatgi *et al.*, 2001; Tang *et al.*, 2005)

Nck-mediated actin assembly stimulated by Tir.

An early hint of the mechanism of pedestal formation induced by EPEC was the observation that EPEC Tir becomes phosphorylated on tyrosine residue 474 (Y474) in the C-terminal domain, and replacement of residue Y474 by phenylalanine severely impairs actin assembly (DeVinney *et al.*, 2001; Kenny, 1999). Distinct host cell kinases have been implicated in Tir phosphorylation. Phillips *et al.* have shown that when Tir is first delivered to host cells using an EPEC strain lacking intimin and then subsequently clustered, c-Fyn is responsible for phosphorylating Tir (Phillips *et al.*, 2004). Contrastingly, Swimm *et al.*, utilizing an EPEC strain expressing both intimin and Tir, found that Arg, Abl, or Tek kinases, recruited to Tir by recognition of a proline-rich region in the Tir N-terminus, are capable of phosphorylating Tir Y474 (Swimm *et al.*, 2004).

Regardless of which kinase is responsible for phosphorylating Y474, this event creates a binding site for the adaptor Nck, which is recruited to EPEC pedestals in a Y474-dependent fashion (Campellone *et al.*, 2002; Gruenheid *et al.*, 2001). Nck is involved in a variety of eukaryotic processes including formation of the immunological synapse, generation of the podocyte intracellular junction in the kidney, photoreceptor axon guidance and targeting in *Drosophila*, and actin filament formation and cell motility (Davis, 2002; Jones *et al.*, 2006; Rao, 2005; Rivera *et al.*, 2006; Tryggvason *et al.*, 2006; Verma *et al.*, 2006). Nck is comprised of three tandem SH3 (Src homology-3) domains and a C-terminal SH2 domain. Each of the three SH3 domains are capable of binding proline-rich sequences in a variety of actin cytoskeletal proteins, though some domains exhibit a preference for specific binding partners (for examples, see Figure 2; Wunderlich

et al., 1999). The Nck SH3 domains activate N-WASP in vitro (Rohatgi *et al.*, 2001), and artificial clustering of the three of the Nck SH3 domains at the plasma membrane of transfected mammalian cells results in localized actin assembly, indicating that these domains can stimulate actin polymerization (Rivera *et al.*, 2004). In the case of EPEC, clustering of Nck SH3 domains at the plasma membrane is promoted by the Nck SH2 domain (Gruenheid *et al.*, 2001), which is capable of binding to a 12-amino acid peptide encompassing phosphorylated Y474 (Campellone *et al.*, 2002). Indeed, clustering of this peptide is sufficient to stimulate actin assembly in cultured cells, and latex beads coated with this peptide, when added to *Xenopus* egg extracts, are able to promote the accumulation of filamentous actin (Campellone *et al.*, 2004). Interestingly, EPEC Tir mediated actin assembly mechanistically resembles actin assembly induced by the pathogen vaccinia virus, which encodes a protein, termed A36R, that becomes phosphorylated on tyrosine residue Y112, thereby creating a docking site for Nck (Frischknecht *et al.*, 1999). For efficient EPEC actin signaling, the requirement for Nck was rigorously documented by demonstrating that EPEC pedestal formation is impaired on Nck-null cells or cells that overexpress the Nck SH2 domain (Gruenheid *et al.*, 2001).

Although the simplest model for EPEC-induced actin assembly envisages the SH3 domains of Nck directly interacting with the PRD of N-WASP, resulting in recruitment and activation of N-WASP at the plasma membrane, N-WASP complementation and recruitment studies resulted in a slightly more complex model in which the Nck-N-WASP interaction was mediated by an additional (and unidentified) host factor. Ectopic expression of N-WASP deletion derivatives in N-WASP-null fibroblasts revealed that, when assessed in a nonquantitative fashion, the PRD was not required for recruitment of

N-WASP to sites of bacterial attachment or for EPEC pedestal formation. An N-WASP derivative comprised only of the WH1 domain and the GBD was recruited to the sites of bacterial attachment, and one lacking the WH1 and part of the GBD domain was unable to function for pedestal formation (Lommel *et al.*, 2001). On the basis of these results, it was postulated that Nck bound to EPEC Tir which interacts with some other eukaryotic protein(s) that recruits and activates N-WASP via the WH1 domain. An initial candidate for this additional host protein was WIP (WASP-interacting protein), which binds the WH1 domain (Ramesh *et al.*, 1997) and plays an essential role in Nck-mediated actin assembly promoted by vaccinia (Moreau *et al.*, 2000). However, EPEC can generate pedestals on WIP-null fibroblasts (S. Snapper, unpublished data) and N-WASP mutants predicted to disrupt WIP binding are still able to function in EPEC pedestal formation (Lommel *et al.*, 2004), but an analogous role might be played by a number of WIP-like proteins, which includes, but is not limited to WICH, WIRE and CR16 (Figure 3).

EPEC triggers actin pedestal formation using Nck-independent pathways

Although depletion of Nck from *Xenopus* egg extracts abolished actin assembly mediated by the Tir Y474 phosphopeptide (Campellone *et al.*, 2004), and EPEC pedestal formation was somewhat impaired on Nck-null fibroblasts (Gruenheid *et al.*, 2001), Campellone *et al.* noted that EPEC retained the ability to generate pedestals on Nck-null cells, albeit of low intensity upon phalloidin-staining and at a frequency four-fold lower than that observed on wild type fibroblasts. N-WASP and Arp2/3 localized to these pedestals, and inhibition of Arp2/3 blocked their formation, indicating that Nck-independent pedestal formation stimulated the well-characterized N-WASP-Arp2/3 pathway. Substitution of

the Y474 to phenylalanine (Y474F) decreased, but did not abolish Tir tyrosine phosphorylation and actin pedestal formation, leading to the identification of a Nck-independent signaling pathway initiated by TirY454, a tyrosine residue twenty amino acids N-terminal to Y474. Interestingly, this pathway resulted in pedestal formation at approximately 5% of wild type levels, and therefore only accounted for a small portion of the Nck-independent signaling initiated by EPEC Tir (Campellone and Leong, 2005).

The arrangement of critical tyrosines Y474 and Y454 in Tir is highly reminiscent of the arrangement of important tyrosines in the A36R protein of vaccinia virus. As mentioned above, residue Y112 of A36R protein becomes phosphorylated, triggering recruitment of Nck and actin assembly. In addition, however, tyrosine residue 132 also becomes phosphorylated and triggers actin polymerization by recruiting a second adaptor, Grb2. Grb2 possess an SH2 domain, which binds phosphorylated Y132, and two SH3 domains, which can stimulate N-WASP-dependent signaling via the PRD (Scaplehorn *et al.*, 2002). Given the parallels between vaccinia and EPEC actin signaling, and the observation that Grb2 is localized to EPEC pedestals (Goosney *et al.*, 2001), it is reasonable to hypothesize that Grb2 could be the adaptor for Y454 in Tir. However, phosphorylated Y454 peptide does not bind to Grb2 *in vitro* and overexpression of the Grb2 SH2 domain does not inhibit pedestal formation on wildtype fibroblasts, so the putative adaptor for this actin signaling cascade remains unknown (Campellone and Leong, 2005).

As noted above, phosphorylation of TirY454 triggers actin pedestal formation at only ~5% of wild type levels, whereas Nck-independent pedestal formation occurs at ~25% of wild type, indicating an alternate route to Nck-independent pedestal formation.

This alternate route depends on Y474, because Nck-independent pedestal formation promoted by a Tir-Y474F mutant occurs only at ~5%, i.e., the efficiency promoted by the Y454 pathway. Thus, EPEC apparently also utilizes an entirely uncharacterized third pathway for pedestal formation, one that depends on Y474 but not Nck (Campellone and Leong, 2005).

Multiple pathways for EPEC pedestal formation: implications for experimental design and interpretation.

EPEC pedestal formation provides an easily manipulated system in which to address the control of actin polymerization at the plasma membrane, and has provided unique insight into the role of Nck in actin signaling. One complicating feature of this experimental system that has only recently been appreciated is that EPEC triggers pedestal formation using at least three pathways: (1) a Y474-dependent, Nck-dependent pathway, that is the best characterized and most efficient; (2) a Y474-dependent, Nck-independent pathway; and (3) a Y454-dependent, Nck-independent pathway. In addition, quantitative analysis of pedestal formation, because it has not been easily automated, requires time consuming visual scoring and has not been routinely performed. Previous experiments, designed and interpreted before alternative Nck-independent pathways were revealed and thus before the benefits of quantitative analyses of pedestal formation were fully appreciated, may have resulted in erroneous conclusions. With these caveats in mind, the studies presented in this thesis involve a quantitative analysis of the role of specific domains of Nck and N-WASP, two host proteins known to play a role in EPEC pedestal formation. Through the use of cells deficient in the production of one or both of these proteins, or the use of

EPEC Tir mutants that selectively stimulate only a subset of the pathways for pedestal formation, specific pathways have been examined in isolation. The results significantly simplify models for Nck-dependent signaling, and provide new insights into Nck-independent pathways of pedestal formation.

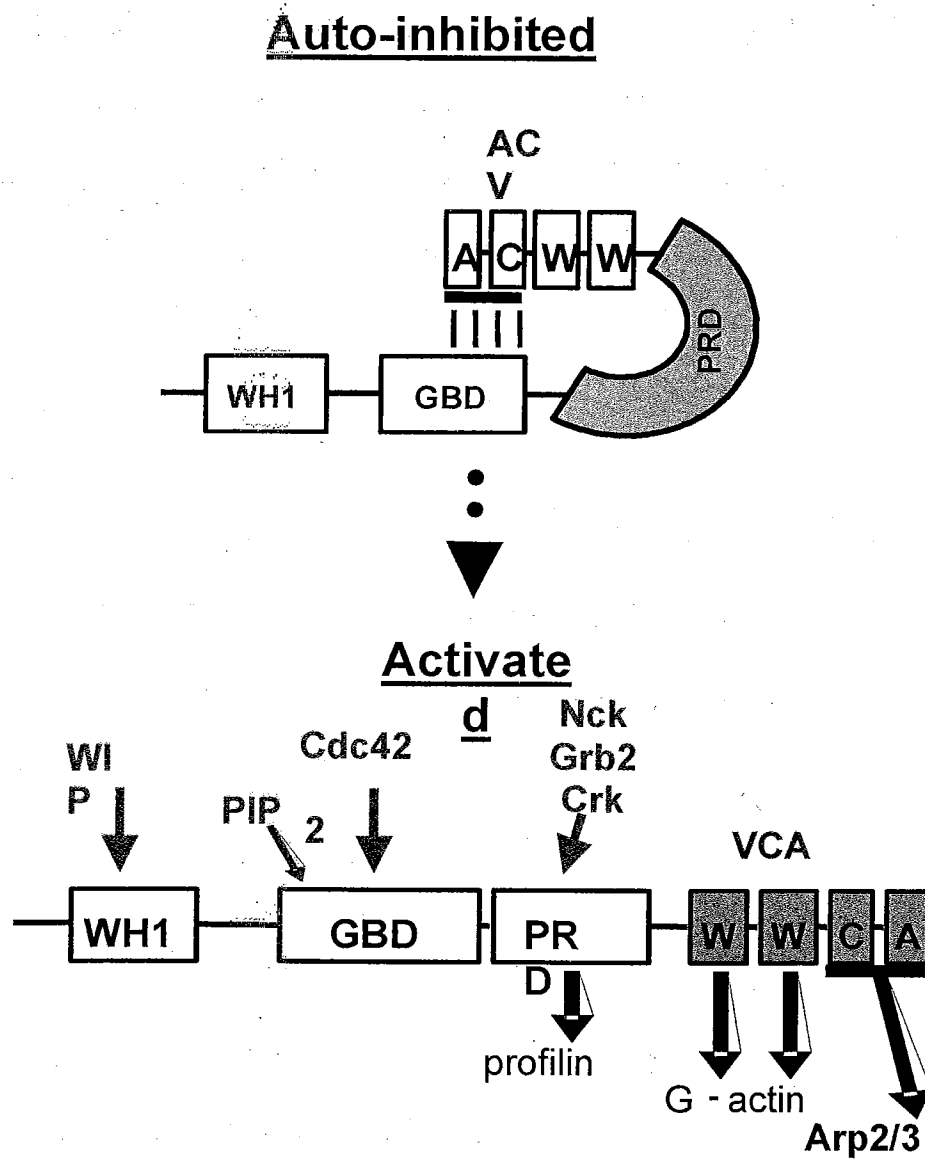


Figure 1. Schematic representation of the autoinhibition and activation of N-WASP.

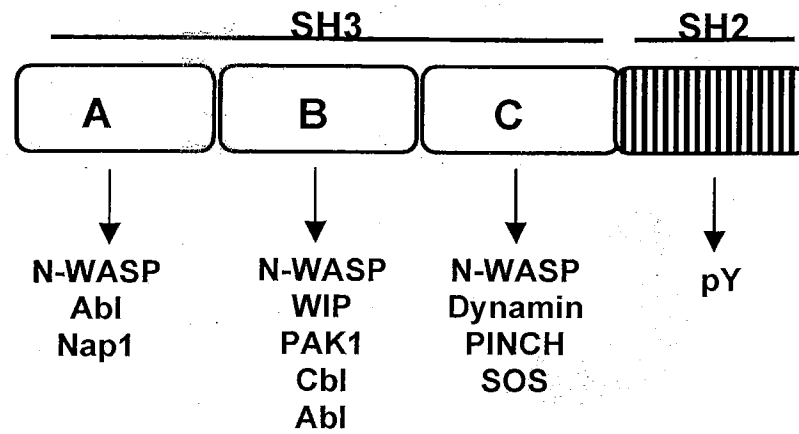


Figure 2. Schematic representation of the domains of Nck and certain respective binding partners.

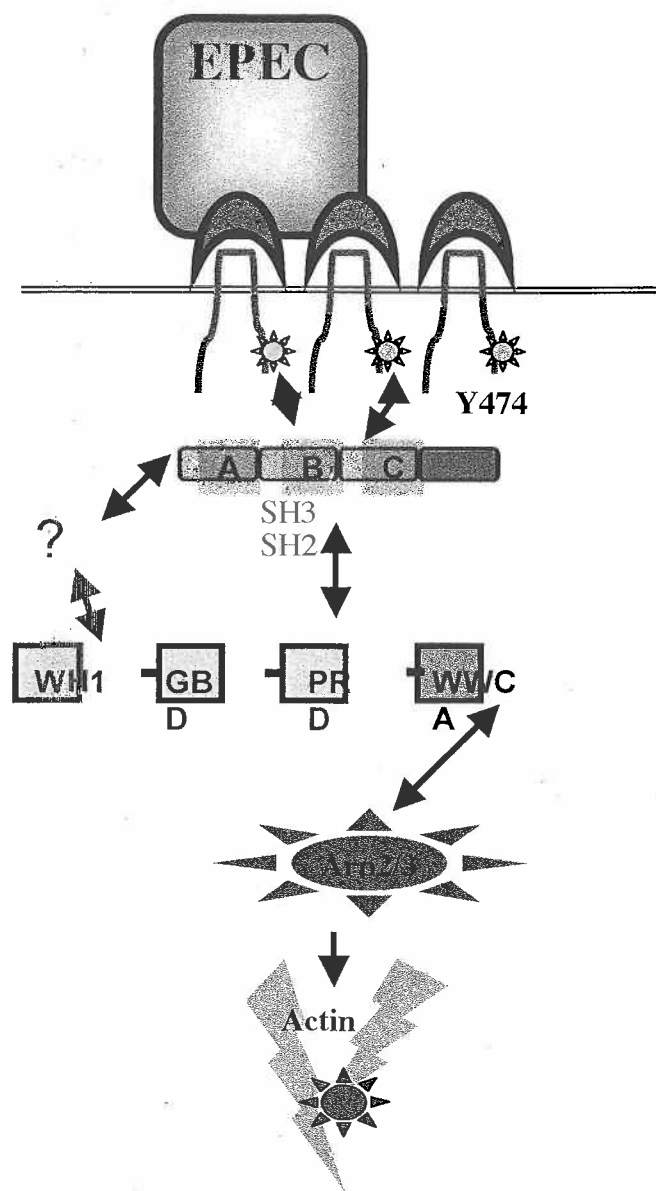


Figure 3. Current model of Y474-dependent, Nck-dependent actin signaling.

CHAPTER II

ANALYSIS OF NCK AND N-WASP SUGGESTS A SIMPLE MODEL FOR ACTIN PEDESTAL FORMATION

Summary

Enteropathogenic *Escherichia coli* (EPEC) generates filamentous actin pedestals beneath sites of bacterial attachment on intestinal epithelial cells by translocating the bacterial effector Tir into host cells, where it becomes clustered at the plasma membrane. Tir triggers at least three pathways for pedestal formation, and the major pathway requires phosphorylation of Tir tyrosine residue 474 (Y474) and the subsequent recruitment of the adaptor Nck and the multi-domain actin nucleation promoting factor N-WASP. Nck encodes an SH2 domain that recognizes the Tir Y474 phosphopeptide and three SH3 domains that can bind the N-WASP proline-rich domain (PRD), leading to activation of N-WASP and actin assembly. However, previous results suggesting that the N-terminal WH1 domain of N-WASP, not the PRD, was essential for pedestal formation led to the hypothesis that the interaction of Nck with N-WASP during actin pedestal formation is indirect. To better understand the Nck-dependent pathway, we have developed experimental systems that allow for interrogation of this pathway in isolation. In doing so, we showed that the second SH3 domain of Nck and the PRD of N-WASP are critical for Nck-dependent actin assembly. These two domains are also critical for direct Nck-N-WASP interaction, suggesting a simple model in which the second SH3 domain of Nck directly interacts with the N-WASP PRD to promote EPEC pedestal formation.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhoea in developing nations, and as a member of the “attaching and effacing” (AE) family of gram-negative enteric pathogens that includes the notable human pathogen, enterohemorrhagic *E. coli* (EHEC) O157:H7, stimulates the production of characteristic AE lesions on the intestinal epithelium to promote bacterial colonization and exact intestinal damage. AE lesions involve the localized destruction of intestinal microvilli, intimate association of the bacterium with the host cell and the accumulation of filamentous (F)- actin in a pedestal-like structure beneath the sites of bacterial attachment (Donnenberg and Whittam, 2001; Kaper *et al.*, 2004). The production of actin pedestals can be recapitulated *in vitro* during infection of cultured mammalian cells (Knutton *et al.*, 1989), rendering EPEC a model system for the evaluation of actin dynamics at the plasma membrane (Celli *et al.*, 2000; Frankel *et al.*, 1998)

For the prototypic EPEC strain, serotype O127:H6, all of the genes required for actin signaling are located on a contiguous 35 kb chromosomal pathogenicity island referred to as the locus of enterocyte effacement (LEE) (Elliott *et al.*, 1998; McDaniel and Kaper, 1997). Encoded by the LEE is a type III secretion apparatus that translocates effector proteins directly into the host cell. The only translocated effector required for EPEC-mediated actin pedestal formation is Tir (translocated intimin receptor, also termed EspE), which is inserted into the plasma membrane in a hairpin-loop configuration with the extracellular central domain serving as a receptor for intimin, a bacterial surface protein (Campellone *et al.*, 2004; de Grado *et al.*, 1999; Deibel *et al.*, 1998; Hartland *et al.*, 1999; Kenny *et al.*, 1997). Binding of intimin-expressing bacteria to Tir results in the

intimate association of the bacterium with the host cell, clustering of Tir, and stimulation of localized actin assembly at the membrane (Caron *et al.*, 2006; Hayward *et al.*, 2006).

EPEC can stimulate actin signaling in at least three different ways using two critical tyrosine residues located in the C-terminus of Tir. The primary pathway of assembly requires tyrosine 474 (Y474), which is phosphorylated by host cell kinases (Phillips *et al.*, 2004; Swimm *et al.*, 2004) thus recruiting the eukaryotic adaptors, Nck1 and Nck2 (which appear to be interchangeable during actin pedestal formation and are herein referred to collectively as Nck) (Campellone *et al.*, 2002; Gruenheid *et al.*, 2001). However, EPEC does not absolutely require Nck since bacteria can stimulate actin accumulation on Nck-null fibroblasts, albeit with less efficiency (Campellone and Leong, 2005). Pedestal formation on these cells can utilize Y474 (in an Nck-independent fashion) or signal through an upstream residue, tyrosine 454 (Y454)-in a manner reminiscent of that seen in vaccinia actin comet tail formation (Scaplehorn *et al.*, 2002), though it is known that, unlike vaccinia, EPEC does not use Grb2 as an alternate adaptor (Campellone and Leong, 2005). Nevertheless, the majority of EPEC-mediated actin assembly relies on phosphorylated Y474 and Nck.

Nck, required for the primary pedestal pathway of EPEC, is central to a variety of signaling pathways that link extracellular stimuli to the assembly of actin filaments, including T cell activation (Davis, 2002), photoreceptor axon guidance and targeting in *Drosophila* (Rao, 2005), cell motility (Rivera *et al.*, 2006) and generation of the podocyte intercellular junction in the kidney (Jones *et al.*, 2006; Tryggvason *et al.*, 2006; Verma *et al.*, 2006). Nck contains a C-terminal SH2 domain, which is capable of binding to specific sequences containing phosphorylated tyrosine residues and thereby linking

tyrosine kinase signaling to actin assembly (Buday *et al.*, 2002), as well as three SH3 domains which are required for stimulation of actin assembly upon artificial clustering of Nck derivatives at the plasma membrane (Rivera *et al.*, 2004).

N-WASP, a ubiquitously expressed member of the Wiskott-Aldrich syndrome protein (WASP)/Scar family of eukaryotic actin nucleation promoting factors (Takenawa and Suetsugu, 2007), is critical for both Nck-dependent and Nck-independent EPEC signaling, because N-WASP-null fibroblasts are unable to promote any EPEC-mediated actin accumulation (Lommel *et al.*, 2001). N-WASP regulates actin assembly via activation of the actin nucleator Arp2/3 complex, which in turn nucleates globular (G-) actin into filamentous (F-) actin. N-WASP is a multi-domain protein comprised of an N-terminal WASP-homology 1 (WH1) domain, followed by a GTPase binding domain (GBD), a proline-rich domain (PRD) and a C-terminal verprolin-connector-acidic (VCA) domain. In its basal state, N-WASP is in an autoinhibited conformation involving the interaction between the VCA and GBD domains, and many regulators of actin assembly, such as the small GTPase Cdc42 and the phospholipid PIP₂, bind N-WASP and disrupt this conformation, allowing the VCA domain of N-WASP to activate the Arp2/3 complex. Nck can interact directly with N-WASP as well, via binding of the Nck SH3 domains to the PRD of N-WASP. Indeed, Nck is capable of activating N-WASP *in vitro*, and maximal activation requires all three Nck SH3 domains (Rivera *et al.*, 2004; Rohatgi *et al.*, 2001).

Artificial clustering of the C-terminus of EPEC Tir in the plasma membrane is sufficient to induce pedestal formation, and a 12-residue Nck-binding Tir Y474 phosphopeptide triggers actin assembly in cell-free *Xenopus* egg extracts when localized

on beads (Campellone *et al.*, 2004). Thus, a simple model for Tir-mediated actin assembly is that Nck, clustered at the membrane through its interaction with Tir, directly binds the PRD of N-WASP, thereby activating it and triggering Arp2/3-mediated actin assembly. Arguing against this model are the observations that the PRD is not required for N-WASP recruitment to sites of bacterial attachment or for complementation of N-WASP-null fibroblasts for EPEC pedestal formation. Instead, the WH1 domain appears critical for EPEC-mediated actin assembly, because an N-WASP derivative lacking the WH1 domain (and the PRD) did not support pedestal formation, and one containing the WH1 and GBD was recruited to sites of bacterial attachment (Lommel *et al.*, 2001).

The presence of multiple pathways of EPEC pedestal formation, which has only recently been appreciated, complicates the analysis of any single pathway. To better understand the Nck-dependent pathway, we have developed experimental systems that allow for interrogation of this pathway in isolation. In doing so, we identified the domains of Nck and N-WASP that are required for Nck-dependent actin assembly, and generate results that support a model in which the second SH3 domain of Nck directly interacts with the N-WASP PRD to promote EPEC pedestal formation.

Materials and methods

Bacterial Strains

Bacterial strains DH5 α and BL21 were used for all cloning and protein purification, respectively. The EPEC strain derived from the parental O127:H6 strain JPN15/pMAR7 (Jerse *et al.*, 1990) was used for all infections.

Bacterial and mammalian cell culture

For routine passage, all bacteria were cultured in LB at 37°C. EPEC was cultured in LB supplemented with ampicillin at 37°C. Nck1/Nck2-null (Nck^{-/-}) and wildtype mouse embryonic fibroblasts (Bladt *et al.*, 2003) were cultured in DMEM (high glucose) plus 10% FBS.

Generation of GST- and GFP-tagged Nck Deletion Derivatives

GST-tagged Nck deletion derivatives were derived using inverse PCR. pGST-SH3(ABC)-SH2 (a kind gift from the Kirschner lab) was used as the template for all PCR reactions. Inverse PCR primers were designed to exclude specific regions of full length Nck. Both the upper and lower primers included an AgeI restriction site for digestion following PCR and prior to ligation with T4 DNA ligase and transformation into competent DH5 α bacterial cells. GFP-tagged SH3(ABC)-SH2 was derived through amplification by PCR of full length Nck from the GST-SH3(ABC)-SH2 template and cloned into the EcoRI and BamHI sites of a pCDNA3 vector containing a GFP tag. The resulting plasmid was used as a template for all inverse PCR reactions (as described above) for the generation of all GFP-tagged Nck deletion derivatives, except GFP-SH3(B)-SH2 and GFP-SH3(C)-SH2. To create GFP-SH3(B)-SH2, the deletion fragment was amplified from GFP-SH3(AB)-SH2 and cloned into the EcoRI and BamHI sites of the GFP-tag containing pCDNA3 transfection vector. A similar strategy was used to generate GFP-SH3(C)-SH2 using pGFP-SH3(BC)-SH2 as the PCR template.

Protein Purification

All GST-tagged Nck derivatives were transformed into electrocompetent BL21 bacterial cells for protein purification. Protein expression was induced when bacterial cultures

reached an $OD_{600}=0.6$ with 1mM IPTG at 37°C for 3 hours. Cells were lysed using various methods, including sonication, commercially available lysis reagents (B-PER II and BugBuster) or cell disruption and supplemented with a commercially available protease inhibitor cocktail (Sigma). The lysed culture was spun at 12,000rpm for 10 minutes, protease inhibitors were added and the supernatant reserved. All reserved supernatants were incubated overnight at 4°C with prepared glutathione-agarose beads (Sigma). Post incubation, the beads were collected via centrifugation and the supernatant reserved at -20°C. The beads were washed three times in cold 1X PBS and proteins eluted five times in 1mL fractions using 5mM reduced glutathione (Sigma) in cold 1X PBS. Proteins were kept at -20°C until needed or at -80°C for long term storage.

Generation of His-tagged N-WASP derivatives.

Hexahistidine tagged N-WASP derivatives were cloned into pBIVT (a kind gift from Neal Silverman) for expression in and purification from Sf9 cells, using the baculovirus system. Infected cells were collected by centrifugation, resuspended in lysis buffer (50mM Tris, pH 8.5, 1% NP-40, 5mM β -2-mercaptoethanol, 1x protease inhibitor cocktail, 1mM PMSF). His-tagged proteins were purified according to published methods.

Transfections and Infections

Nck- null mouse embryonic fibroblasts were grown in six-well plates until ~90% confluence and transfected with 500ng of GFP-tagged plasmid per well for 12 hours using Lipofectamine Plus reagent (Invitrogen). Cells were reseeded onto 12-mm glass coverslips to achieve 50-75% confluency after an additional 24 hours of growth at 37°C in a 5% CO₂ atmosphere. Cells were infected with 2×10^6 EPEC in DMEM+3% FBS +

25mM HEPES pH 7.4 for 3.5 hours at 37°C in a 5% CO₂ atmosphere before being fixed and permeabilized for fluorescence microscopy.

Fluorescence Microscopy

Infected monolayers were fixed in PBS + 2.5% paraformaldehyde for 30 min and permeabilized with PBS + 0.1% Triton X-100 for 5 min. Bacteria were visualized by treatment with DAPI (1µg/mL; Sigma Aldrich) and F-actin was identified with TRITC-phalloidin (1µg/mL; Sigma Aldrich). Positively transfected cells were identified by the GFP expression. Pedestal formation efficiency was quantitated by identifying positively transfected cells with at least five EPEC bound, scoring the number of pedestals formed and calculating the percent of bound bacteria generating pedestals. Ten positively transfected cells meeting the above requirements were examined per experiment and each experiment was repeated at least three times. It should be noted that overexpression of N-WASP derivatives predicted to be constitutively active (WPV, WV, PV) promoted the production of actin punctae in the cell cytoplasm.

Generation of Cell-Free Fibroblast Extracts

To obtain fibroblast extracts cells were processed in a manner similar to that described in Saoudi *et al* with minor modifications (Saoudi *et al.*, 1998). Cells were grown on tissue culture flasks with surface areas of 636 cm² (Corning) until they reached between 70 and 90% confluence. The culture medium was removed and cells were washed with sterile PBS (137mM NaCl, 8mM sodium phosphate, 1.5mM potassium phosphate, 2.7mM KCl, pH 7.4). Cells were trypsinized at 37°C for 5 minutes, collected by centrifugation (10 min, 37°C, 300g), the pellets resuspended in PBS and recentrifuged (10 min, 37°C, 300g). The pellet was resuspended in 10mL permeabilization buffer (80mM Pipes-KOH, pH

6.75, 1mM MgCl₂, 1mM EGTA, 0.5% Triton X-100 (vol/vol) and 10% glycerol (vol/vol) supplemented with 5mM CaCl₂). These cells were incubated for 10 minutes at 4°C, with gentle agitation. Post incubation, additional EGTA was added to a final concentration of 10mM. The extracts were centrifuged (20min, 4°C, 200,000g) in a 80ti rotor in Beckman Coulter Optima L-100 XP Ultracentrifuge. The supernatant was divided into 100μL aliquots, immediately snap frozen in liquid nitrogen and stored at -80°C until further use. For the generation of concentrated N-WASP-null extracts, a similar method was applied though the cell pellet was resuspended in only 1mL of lysis buffer and centrifuged (20min, 4°C, 200,000g) in a Airfuge ultracentrifuge (Beckman). The supernatant was divided into 50μL aliquots and snap frozen as described above.

Fibroblast extract assays

GST-tagged recombinant Nck deletion derivatives were added to a final concentration of 7nM in 100μL of Nck-null extract or His-tagged N-WASP deletion derivatives were added to a final concentration of 50nM in 100μL of N-WASP-null extract and incubated on ice for one hour. 5.5μL of all extracts were supplemented with 0.5μL rhodamine-labeled rabbit skeletal muscle actin (1mg/mL, Cytoskeleton, Inc) and 0.2μL of CSF energy mix (150mM creatine phosphate, 20mM ATP and 20mM MgCl₂). The twelve amino acid Tir peptide containing the phosphorylated tyrosine 474 was bound to streptavidin-labeled 1μm latex beads (Sigma) as previously described (Campellone *et al.*, 2004) diluted 10-fold into supplemented extracts and incubated, in the dark, for 30 minutes at room temperature prior to examination for actin assembly using epifluorescent microscopy. The reaction volumes were doubled for evaluation by flow cytometry.

Gel Overlay

His-tagged N-WASP derivatives were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and blocked with PBS+5% milk for 30 minutes at room temperature. The membrane was then probed with 1 μ g/ml GST-tagged Nck derivatives in PBS+0.2% BSA for two hours at RT or at 4°C overnight, and blocked again in PBS+5% milk for thirty minutes at RT. The GST-Nck derivative bound to the membrane was detected by goat anti-GST antibody (1:1,000, Neomarkers) and sheep anti-goat IgG conjugated to alkaline phosphatase (1:10,000, Sigma). The His-tagged N-WASP derivatives were detected in a similar manner using mouse anti-His antibody (1:3000) and sheep anti-mouse IgG conjugated to alkaline phosphatase (1: 10,000, Sigma).

ELISA

Ninety-six-well microtiter plates (Corning) were coated with His-tagged N-WASP derivatives at 16nM in PBS at 4°C overnight. Wells were blocked in PBS+5% milk BSA for 2 hours at 20°C and probed with GST-tagged Nck derivatives ranging from 3.25nM to 135nM in PBS+ 0.1% BSA for 3 hours at 20°C. Wells were washed in PBS and blocked again in PBS+5% milk. Bound GST-tagged Nck derivatives were detected by rabbit anti-GST antiserum (1:1000, Amersham) and sheep anti-goat IgG conjugated to horseradish peroxidase (1:10,000, Sigma).

Western blotting

Twenty microliters of wildtype, Nck-null and supplemented Nck-null extracts were resuspended in twenty microliters of 2x SDS-PAGE buffer, subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Both endogenous Nck (42kD) and GST-tagged

Nck (67kD) were detected using a rabbit anti-Nck antibody (1:1,000, Neomarkers) and sheep anti-rabbit IgG conjugated to alkaline phosphatase (1:10,000, Sigma).

Results

The central SH3 domain of Nck, SH3(B), is essential for Tir-mediated pedestal formation on cultured fibroblasts.

In keeping with an important role for Nck during actin assembly triggered by EPEC, this bacterium does not efficiently form pedestals on Nck-null fibroblasts (Gruenheid *et al.*, 2001). One or more inefficient pathways of pedestal formation function independently of Nck, reflected by retention of the ability of EPEC to generate weakly staining foci of F-actin at low (~20-25%) frequency on these cells (Campellone and Leong, 2005), but complementation with GFP-Nck results in a dramatic increase in the level of actin pedestal formation, equivalent to wild type (Gruenheid *et al.*, 2001). Indeed, in the current study, we found that ~76% of EPEC bound to wild type fibroblasts generated F-actin foci detectable by phalloidin staining, in contrast to the ~10-15% of bound bacteria that generated pedestals on untransfected Nck-null cells (Campellone and Leong, 2005) or Nck-null cells ectopically expressing GFP (Figure 1). When Nck-null cells ectopically expressed GFP-Nck, 80-85% of bound bacteria generated pedestals cells (Figure 1), as previously observed (Campellone and Leong, 2005).

The SH2 domain, which binds to the Tir Y474 phosphopeptide, is essential for Nck function in EPEC pedestal formation (Gruenheid *et al.*, 2001; Kenny, 1999). To examine the activity of Nck derivatives that, in addition to the SH2 domain, contain only a single SH3 domain, pedestal formation was scored after EPEC infection of Nck-null cells ectopically expressing GFP fusion proteins that contain the SH2 domain and one of

the three SH3 domains. GFP-SH3(A)-SH2, which contains the first SH3 domain, provided no detectable Nck function, as the percentage of pedestals on transfected cells was not distinguishable from that on GFP-transfected cells (Figure 1), whereas GFP-SH3(B)-SH2 or GFP-SH3(C)-SH2, containing the second or third SH3 domains, respectively, demonstrated Nck function for pedestal formation. Approximately 46% of bacteria generated detectable F-actin foci on Nck-null cells expressing GFP-SH3(C)-SH2; the partial nature of this complementation was also reflected in the observation that less than 8% of bacteria were associated with intensely staining pedestals (Figure 1, arrowheads). In contrast, GFP-SH3(B)-SH2 fully complemented Nck-null cells for pedestal formation, because the formation efficiency of detectable pedestals (74%) and of intensely-stained pedestals (59%) was indistinguishable from fibroblasts expressing full length GFP-Nck (82% and 56%, respectively; Figure 1). We conclude that, in conjunction with the Nck SH2 domain, the second Nck SH3 domain can apparently confer full function, the third domain partial function, and the first domain no function for EPEC-mediated, Nck-dependent actin pedestal formation.

The central SH3 domain of Nck, SH3(B), is essential for actin assembly initiated by TirY474 phosphopeptide in cell-free extracts.

Latex beads coated with a twelve amino acid Tir peptide encompassing the critical phosphorylated tyrosine 474 stimulate actin assembly in cell-free *Xenopus* egg extracts and depletion of Nck from these extracts eliminates actin assembly (Campellone *et al.*, 2004), indicating that the only mode of Tir-mediated actin assembly in these extracts is Nck-dependent. To evaluate whether mammalian cell free extracts would behave in a

similar manner, extracts were generated from wildtype and Nck-null mouse embryonic fibroblasts. After the addition of the Tir peptide-coated beads to wildtype cell extracts supplemented with rhodamine actin, distinct bright fluorescent foci appeared around most beads (Figure 2A), although few were in the form of “comet tails” that are often observed in *Xenopus* egg extracts challenged with the same beads (Campellone *et al.*, 2004). In contrast, no fluorescent foci were observed around uncoated beads or beads in Nck-null cell extracts (Figure 2A). To test whether the lack of foci was due to the absence of Nck, 1nM to 10nM recombinant full length GST-Nck was added to Nck-null extracts. As little as 4nM GST-Nck (also referred to as GST-SH3(ABC)-SH2) was capable of promoting a detectable fluorescent signal (data not shown), and at 7nM GST-Nck, bead-associated fluorescence was even more striking than that observed in Nck-proficient extracts (Figure 2A). Immunoblotting with anti-Nck antibody suggested that Nck is present in wild type cell extracts at approximately 7nM (Supplementary Figure 1); the more robust signal associated with the addition of 7nM GST-Nck could be due to multimerization conferred by the GST tag. As expected, replacement of tyrosine 474 of the 12-residue peptide with phenylalanine abrogated actin assembly in this assay (data not shown), and the addition of 2 μ M cytochalasin D, an inhibitor of actin assembly, diminished bead-associated fluorescence (Figure 2B). Neither the SH2 domain nor the SH3 domains could promote actin assembly in isolation, because the addition of either GST-SH3(ABC) or GST-SH2 to Nck-null extracts did not promote actin accumulation on beads coated with the Tir peptide any better than did the addition of GST alone (data not shown).

To analyze the efficiency of actin assembly in a quantitative fashion, we subjected the beads to flow cytometry. Beads were gated by forward and side scatter and bead-associated rhodamine fluorescence was measured to estimate the percentage of beads that trigger the formation of F-actin. We defined a high threshold fluorescence value that excluded 98.6% of the beads incubated in Nck-null extracts (Figure 2C) and 98.4% of uncoated beads incubated in Nck-supplemented extracts (Figure 2B). When the beads were incubated in wildtype extract, more than 15% of beads were positive for F-actin (Figure 2C), and when incubated in Nck-supplemented extracts, more than 95% of beads were above threshold, consistent with the robust actin assembly visualized by fluorescent microscopy (Figure 2A,C).

To determine if any combination of the SH2 domain and two SH3 domains could promote Tir-mediated actin assembly *in vitro*, we generated GST-SH3(AB)-SH2, GST-SH3(AC)-SH2, and GST-SH3(BC)-SH2, lacking the third, second, and first SH3 domains, respectively. Attachment of these recombinant proteins to peptide-coated beads indicated that each of the derivatives retained a functional SH2 domain (data not shown). GST-SH3(AC)-SH2, when added to Nck-null extracts, could not trigger actin polymerization on peptide-coated beads (Figure 2C), suggesting that the second SH3 domain, SH3(B), is required for function. In contrast, both GST-SH3(AB)-SH2 and GST-SH3(BC)-SH2 were capable of providing Nck function to Nck-null extracts. GST-SH3(AB)-SH2 and GST-SH3(BC)-SH2 each promoted actin assembly on the vast majority (78% and 87%, respectively) of beads, indistinguishable from the percentage (85%) of positive beads after incubation with full-length GST-Nck-supplemented extracts

(Figure 2C). These results are consistent with the model that the second domain, SH3(B), plays the most important role in EPEC Tir-mediated actin assembly *in vitro*.

To further investigate the activity of SH3(B), GST-tagged derivatives comprised of the SH2 domain and individual SH3 domains were added to Nck-null extracts and challenged with latex beads coated with the Tir phosphopeptide. No actin assembly was observed in these extracts (Figure 2D; hatched bars), and increasing the concentration of recombinant GST-Nck protein to 10nM had no effect (data not shown). Rohatgi *et al* previously demonstrated by far Western and pulldown assays that the individual Nck SH3 domains did not bind to N-WASP as efficiently as full length Nck (Rohatgi *et al.*, 2001). To determine if increasing the concentration of N-WASP to higher than endogenous levels might reveal activity of the GST Nck proteins containing on a single SH3 domain, 100nM of recombinant His-tagged N-WASP was added to these Nck-null extracts supplemented with the GST-tagged derivatives. When these N-WASP-supplemented, Nck-null extracts were challenged latex beads coated with Tir phosphopeptide, extracts supplemented with GST-SH3(A)-SH2 were still unable to promote any actin accumulation around the bead (Figure 2C, black bar). N-WASP-supplemented, Nck-null extracts containing GST-SH3(C)-SH2 were only mildly positive for actin assembly, as the addition of recombinant N-WASP increased the percentage of actin positive beads from 4% to 9% (Figure 2C, black bar). Supplementation of the extracts with even higher concentrations of GST-SH3(A)-SH2, GST-SH3(C)-SH2, or recombinant N-WASP had no effect on actin assembly (data not shown). Notably, GST-SH3(B)-SH2, when added to N-WASP-supplemented, Nck-null extracts, restored actin assembly to levels near those seen in Nck-null extracts supplemented with full length

GST-Nck, approximately 73% (Figure 2C, black bar). Thus, the activities of recombinant Nck derivatives in promoting Tir-mediated actin assembly *in vitro*, are entirely consistent with the assessment of Nck function by complementation of Nck-null cells: of the three Nck SH3 domains, the second confers the most function in Nck-dependent signaling by EPEC, the third domain confers less function, and the first domain confers the least function.

The ability of Nck derivatives to function in Tir-mediated actin assembly correlates with their ability to interact with N-WASP

To determine if the ability of Nck derivatives to provide function in actin pedestal formation correlated with their ability to interact with N-WASP, GST-Nck derivatives containing the SH2 domain and one of the SH3 domains were assayed for the ability to bind recombinant N-WASP in far Western assays. Full-length GST-Nck bound to N-WASP in this assay (Figure 3A; Rohatgi *et al.*, 2001). GST-SH3(B)-SH2, which contains the second SH3 domain and showed function in the *in vitro* actin assembly assays, bound to full-length N-WASP in this assay (Figure 3A), albeit apparently not as well as did full length Nck, as visually assessed for band intensity. As expected for an SH3 domain-mediated interaction, assaying binding to deletion derivatives of N-WASP revealed that binding was dependent on the N-WASP proline-rich domain (PRD), but not the WH1 domain, GTPase binding domain (GBD), or VCA domain (Figure 3A, “WGV”). GST-SH3(C)-SH2, which contains the third SH3 domain and showed low level function in actin assembly assays, also bound to N-WASP in a PRD-dependent manner, but with less apparent efficiency than GST-SH3(B)-SH2 (Figure 3A). Finally, GST-

SH3(A)-SH2, which contains the first SH3 domain and showed no activity in Tir-mediated actin assembly, did not detectably bind N-WASP.

To determine the relative binding efficiencies of the Nck derivatives in a somewhat more quantitative fashion, microtiter wells were coated with recombinant N-WASP and then probed with increasing concentrations of GST fusion proteins, which were revealed by ELISA using anti-GST antibody. Full-length bound well to N-WASP as an N-WASP coating concentration of approximately 7nM gave a half-maximal signal (Figure 3B). GST-SH3(B)-SH2 bound to immobilized His-N-WASP with a half-maximal coating concentration of about 32nM, and GST-SH3(C)-SH2 bound immobilized N-WASP with a half-maximal coating concentration of about 88nM. GST-SH3(A)-SH2, which lacked activity in actin assembly, did not detectably bind N-WASP in this assay, as binding was not significantly different from binding by GST-SH2 (Figure 3B). These assays indicate that the ability of Nck derivatives containing the SH2 domain and one SH3 domain to bind to the N-WASP PRD *in vitro* correlates well with their hierarchy of function in promoting actin pedestal on cultured cells or actin assembly in cell-free extracts.

The N-WASP PRD, not the WHI domain, plays a primary role in EPEC-mediated actin assembly.

EPEC-induced actin pedestals were previously observed on N-WASP-null fibroblasts expressing an N-WASP deletion derivative lacking the PRD (Lommel *et al.*, 2001).

However, a quantitative assessment was not performed in that study, and analysis of N-WASP domains required for a particular (e.g., Nck-dependent) pathway of actin

assembly is complicated by the recent discovery that Tir triggers multiple pathways of actin pedestal formation (Campellone and Leong, 2005). Given that the relative frequencies of the different actin assembly pathways are distinguishable, we sought to assess the contribution of the various domains of N-WASP to EPEC-mediated actin assembly in a quantitative fashion by measuring pedestal formation on N-WASP-null fibroblasts expressing various GFP-tagged N-WASP derivatives. When wildtype mouse embryonic fibroblasts were infected with EPEC, approximately 70% of attached bacteria generated actin pedestals, consistent with previously published work (data not shown; (Campellone and Leong, 2005). Untransfected N-WASP-null fibroblasts, or transfected N-WASP-null cells expressing GFP, were unable to promote any actin accumulation beneath bacteria, while (as previously observed; Lommel *et al.*, 2001) the expression of full-length GFP-N-WASP in these cells fully complemented this deficiency (Figure 4). An N-WASP derivative lacking the GBD retained the ability to fully complement the N-WASP-null fibroblasts for EPEC-mediated pedestal formation, as did a derivative lacking the WH1 domain (Figure 4), indicating that, contrary to previously published work (Lommel *et al.*, 2001), the WH1 domain, like the GBD, does not appear to be essential for pedestal formation. Furthermore, N-WASP derivatives that lacked only the PRD, or both the PRD and the GBD were unable to fully complement the N-WASP defect: pedestals were generated at approximately one-quarter the wildtype frequency (Figure 4). The residual 25% activity of the N-WASP derivative lacking the PRD appeared to depend on the WH1 domain, because a derivative lacking both the PRD and the WH1 domain had no activity. We have previously shown that the Nck-independent pathways promote pedestal formation at approximately 25% of wildtype levels, characterized by

pedestals that stain less intensely for F-actin than those observed in the primary, more efficient Nck-dependent pathway. Therefore, it is tempting to speculate that the 25% residual activity of N-WASP derivatives retaining the WH1 is due to Nck-independent pedestal formation, and the full activity by derivatives lacking the only the WH1 domain or GBD reflects the primary (and more efficient) Nck-dependent pathway. Curiously, a derivative consisting of only the PRD and the VCA, which might be predicted to retain full activity, did not complement the N-WASP-null cells at all (Figure 4), though we were unable to determine if the lack of activity conferred by the PRD-VCA derivative was due to misfolding or the potential for this protein to act as a dominant negative inhibitor of Arp 2/3.

To assess the role of the N-WASP domains in Tir-mediated actin assembly in cell-free extracts, we added beads coated with the Tir Y474 phosphopeptide to extracts generated from N-WASP-null fibroblasts supplemented with rhodamine actin and recombinant N-WASP derivatives at a concentration of 100nM, which we estimate to be the approximate endogenous levels of N-WASP (Suzuki *et al.*, 1998). Unsupplemented extracts were largely incompetent for assembly, with no visual evidence of bead-associated assembly and only 1.6% of beads with fluorescence above the set threshold upon analysis by flow cytometry (Figure 5). Extracts supplemented with full length N-WASP promoted assembly on a sizable fraction of beads coated with the Tir phosphopeptide, as assessed visually, and flow cytometric analysis revealed approximately 20% of beads positive for actin accumulation, a level consistent with that observed in wildtype fibroblast extracts (Figures 2 and 5). A derivative lacking the GBD ('WPV') functioned indistinguishably from full length N-WASP in this assay, consistent

with the full function of an analogous GFP derivative in complementation of N-WASP cells for pedestal formation (Figure 5). An N-WASP derivative lacking the PRD ('WGV') was largely defective for Tir-mediated actin assembly, as less than 4% of beads were scored positive for actin assembly, indicating that the PRD plays an essential role in this assay system and consistent with our previous analysis implicating this domain. (The level of assembly was slightly higher than that observed for unsupplemented N-WASP-null extracts (1.6% beads scored positive); it is possible that this marginal activity could be due to low level Nck-independent assembly occurring in these extracts, which were prepared in a concentrated fashion; see Materials and Methods). In addition, when added to an N-WASP-null extract supplemented with an N-WASP derivative lacking the WH1 and GBD, i.e., one that carried only the PRD and VCA ('PV'), 19% of peptide-coated beads were positive for actin assembly, a level statistically indistinguishable from beads incubated in wildtype extracts or N-WASP-null extracts supplemented with full length His-N-WASP (Figure 5). This result suggests that observation that the analogous N-WASP derivative containing only the PRD and VCA did not function in the transfection assay described above (see Figure 4) is not due to the absence of a specific N-WASP domain, but may be due to an unknown regulator that is active in intact cells but not in extracts, or to misfolding of the GFP fusion protein. Importantly, the results indicate that the only domains of N-WASP essential for actin assembly mediated by the TirY474 phosphopeptide are the PRD and VCA domain.

The PRD of N-WASP is an essential element in Tir-mediated, Nck-dependent actin assembly *in vitro*.

The N-WASP PRD is an essential domain for *in vitro* actin assembly triggered by the TirY474 phosphopeptide. Although this finding suggests that the PRD is essential for Nck-mediated actin assembly stimulated by EPEC, we cannot formally rule out the possibility that a non-Nck adaptor functions in this assay. To unequivocally link the N-WASP domain analysis to Tir-mediated, Nck-dependent actin assembly, we took advantage of the observation that Nck-null extracts supplemented with the inactive Nck fusion, GST-SH3(A)-SH2, were incapable of promoting actin assembly on beads coated with Tir Y474 phosphopeptide, even when the extracts were supplemented with recombinant full length N-WASP (Figure 2D). In contrast, when (and only when) Nck-null extracts were supplemented with the active Nck derivative, GST-SH3(B)-SH2, exogenously added N-WASP promoted assembly on coated beads (see Figure 2D). These observations provided a means to interrogate different N-WASP derivatives for function in an experimental system in which only the Nck-dependent (in this case, GST-SH3(B)-SH2-dependent) pathway is active. As previously shown (see Figure 2D), the addition of full-length recombinant N-WASP to Nck-null extracts supplemented with GST-SH3(B)-SH2 resulted in robust, bead-associated actin assembly, with approximately 56% of beads scored F-actin positive by flow cytometry, compared to 2.2% positive when no exogenous N-WASP was added (Figure 2D). Deletion of the WH1 domain had no significant effect on this activity, consistent with all of our previous analyses. Deletion of the GBD had a partial effect on activity, as approximately 30% of beads were scored positive (Figure 6); it is unclear how to interpret this (less than two-fold) effect, as this same deletion showed full function in the complementation of N-WASP-null extracts (Figure 5). Most strikingly, deletion of the PRD resulted in a total loss of activity, with

only ~4% of beads positive for actin accumulation; whereas a derivative containing only the PRD and VCA showed extremely robust activity, with 88% beads positive for F-actin (Figure 6). While there are potential explanations for the greater than wild type activity (see Discussion), these results unequivocally link the N-WASP PRD to Nck-dependent actin assembly triggered by Tir Y474 phosphopeptide.

Discussion

A previous analysis of the N-WASP domains required for EPEC pedestal formation, performed soon after the critical role for Nck in EPEC pedestal formation was established, reported that the N-WASP WH1 domain, rather than the PRD (Lommel *et al.*, 2001). This finding led to the hypothesis that during pedestal formation Nck does not directly interact with N-WASP, but rather recruits (and activates) this factor indirectly, by binding a second (WH1-binding) factor. Since that time, it has been revealed that EPEC pedestals can be generated via (at least) three pathways, of which the most efficient is dependent on Nck. The observation that all three pathways converge on N-WASP (Campellone and Leong, 2005; Lommel *et al.*, 2001) complicates N-WASP domain analyses because different domains may play disparate roles in each of the pathways.

To better characterize the Nck-dependent pathway, we first investigated the relative importance of each of the three Nck SH3 domains by testing the ability of Nck derivatives to complement Nck-null fibroblasts for pedestal formation. Transfected derivatives GFP-SH3(AC)-SH2 (data not shown) and GFP-SH3(C)-SH2 partially complemented Nck-null fibroblasts and GFP-SH3(B)-SH2 fully complemented Nck-null cells for EPEC pedestal formation. Assessment of Nck function by reconstituting Nck-

dependent actin assembly in cell-free mammalian extracts indicated that the Nck SH3(B) domain played a central role in this experimental system: deletion of the Nck SH3(B) domain, but not the SH3(A) or SH3(C) domains, entirely abrogated assembly, and a derivative that contained only the Nck SH2 and SH3(B) domains promoted assembly when the extracts were supplemented with exogenous N-WASP. In contrast, an analogous derivative carrying the SH3(A) domain had no detectable activity, and one carrying the SH3(C) domain showed only marginal activity, though the difference between the two was statistically significant, consistent with the low level of robust pedestal formation observed on cells transfected with the analogous derivative. Thus, both assays suggest that of the three Nck SH3 domains, the central SH3(B) domain plays the most important and the N-terminal SH3(A) domain the least important role in Tir-mediated actin assembly.

The ability of a Nck derivative encompassing only the SH2 and the second SH3 domains to provide robust actin assembly activity contrasts with previous deletion analyses of Nck. Whereas the Nck SH2 domain is dispensable for maximal activation of purified N-WASP *in vitro*, all three SH3 domains of Nck are required (Rohatgi *et al.*, 2001). Similarly, artificial clustering of the three SH3 domains of Nck at the membrane of 293T cells resulted in actin polymerization, but removal of any of the SH3 domains almost completely abrogated assembly (Rivera *et al.*, 2004). Thus, the requirements for Nck during actin pedestal formation appear less stringent than, and might be different from, those in these other Nck-dependent actin assembly assays. Engagement of the Nck SH2 domain by the Tir Y474 phosphopeptide might enhance the actin signaling activity of the SH3 domains, although the conformation of isolated Nck SH2 domain does not

appear to be significantly altered by binding of the Tir Y474 phosphopeptide (Frese *et al.*, 2006). Alternatively, although little quantitative information is available concerning the density of Tir beneath bound bacteria, by clustering Tir via intimin, EPEC may promote a particularly high local concentration of Nck. The fact that Nck localized by Tir will be in proximity to the plasma membrane, and thus near other potential activators of N-WASP such as phosphoinositides, may also contribute to the ability to detect actin assembly activity of Nck derivatives lacking one or more SH3 domains.

Analysis of the interaction of the Nck SH3 domains with N-WASP and of the domains of N-WASP critical for Tir-mediated actin assembly were consistent with the model that actin assembly is a result of the direct interaction of these two proteins. First, the relative abilities of the Nck SH3 domains to bind N-WASP in vitro closely correlated their relative activity in promoting actin assembly, i.e., of the three domains, SH3(B) bound most efficiently and SH3(A) worst. This binding, as previously shown, required the N-WASP PRD, and ectopic expression of N-WASP derivatives in N-WASP- null cells implicated this same domain, because derivatives lacking the PRD generated pedestals at only 25% of wild type levels. Evidence for a critical role for the PRD was also generated using actin assembly in N-WASP-null cell free extracts supplemented with N-WASP derivatives. Finally, by utilizing conditions under which actin assembly was demonstrably dependent on the Nck SH3(B) domain (i.e., dependent on the addition of GST-SH3(B)-SH2), we showed that the N-WASP PRD-VCA (i.e., the PRD in combination with the effector VCA domain) could promote high level actin assembly.

Clearly, domain deletion analyses can be misinterpreted due to aberrant folding or, particularly in the case of modular proteins with multiple regulatory domains, aberrant

regulation of the deletion derivative. Some of the analyses of the N-WASP deletion derivatives presented here may reflect such pitfalls. For example, ectopic expression of a GFP fusion to N-WASP PRD-VCA did not show any activity in pedestal formation, in spite of the fact that an analogous recombinant GST derivative demonstrated good activity in cell-free Tir-mediated actin assembly assays. It is possible that the GFP fusion was not folded properly; alternatively, the fact that a PRD-VCA derivative is predicted to be constitutively active due to the absence of a GBD-VCA interaction that keeps N-WASP in an inactive state, may lead to unpredictable results when expressed at high levels in cultured cells. The possibility that a PRD-VCA derivative is in a constitutively active state may also contribute to the observation that the GST fusion protein containing this fragment resulted in higher levels of actin assembly in cell-free extracts than even that that promoted by full-length N-WASP. Nevertheless, the robust Tir-mediated actin assembly in extracts in which the functional versions of Nck and N-WASP are GST-SH3(B)-SH2 and PRD-VCA, containing the very domains that mediate efficient Nck-N-WASP interaction, strongly supports the hypothesis that this pathway of actin assembly involves a direct interaction between the two proteins.

The results presented here contrast with a previous domain analysis of N-WASP, which concluded that the WH1, rather than the PRD, is essential for N-WASP recruitment in EPEC pedestal formation (Lommel *et al.*, 2001). Nevertheless, several aspects of the two studies are compatible. For example, both studies report that deletion of the N-WASP PRD does not abolish pedestal formation. We found, however, an approximately four-fold loss of efficiency, a defect that might only be detected upon quantitation of pedestal formation, a step that was not performed in the previous study. In addition, both

studies found that the WH1 domain plays a role in pedestal formation, although we report here that the contribution of this domain is relatively small (e.g., sufficient to generate pedestals at ~25% of wild type levels), a level consistent with a role for this domain in Nck-independent pedestal formation. Thus, one important development in more recent studies that has facilitated interpretation of results has been quantitation of pedestal formation, whereas prior to the identification of multiple pathways, this step was not routinely performed. In addition, in this study, the use of Nck-null cells, as well as extracts derived from those cells, has permitted evaluation of specific subsets of the pathways of pedestal formation, and modification of a cell-free Tir-mediated actin assembly assay has allowed for the exclusive interrogation of the Nck-dependent pathway. Given that virtually nothing is known concerning Nck-independent pedestal formation, which constitutes a significant pathway, at least on mammalian cells in culture, techniques to isolate specific Tir-mediated assembly pathways may provide new insight into the control of actin assembly at the eukaryotic plasma membrane.

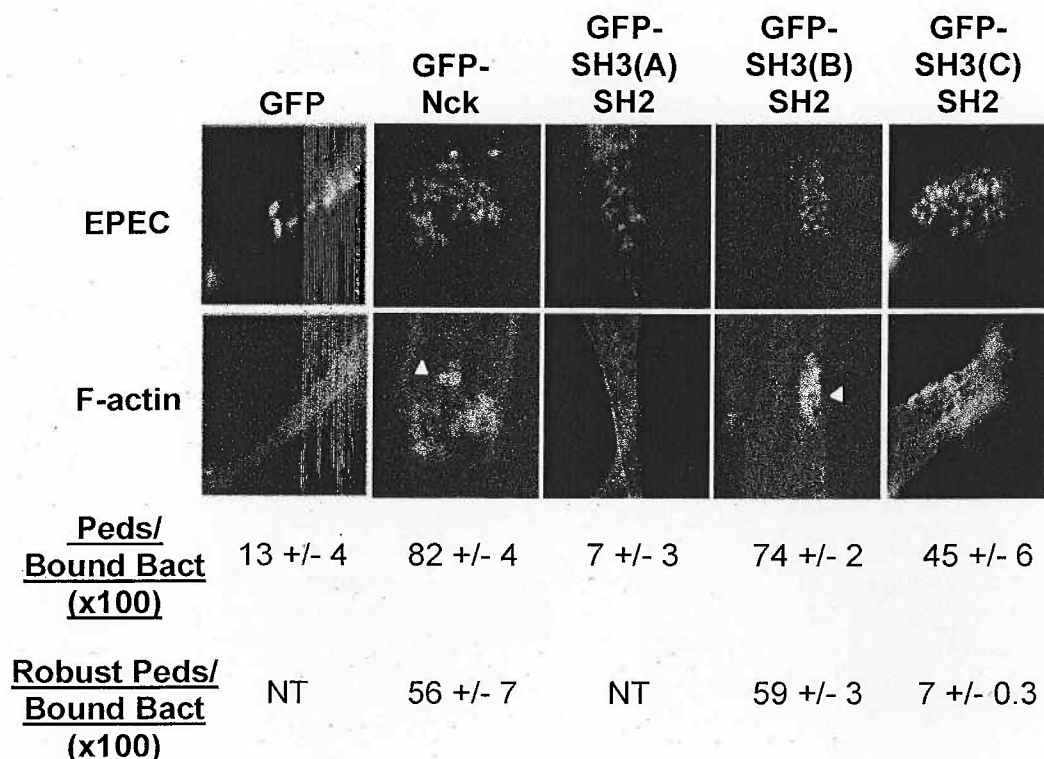
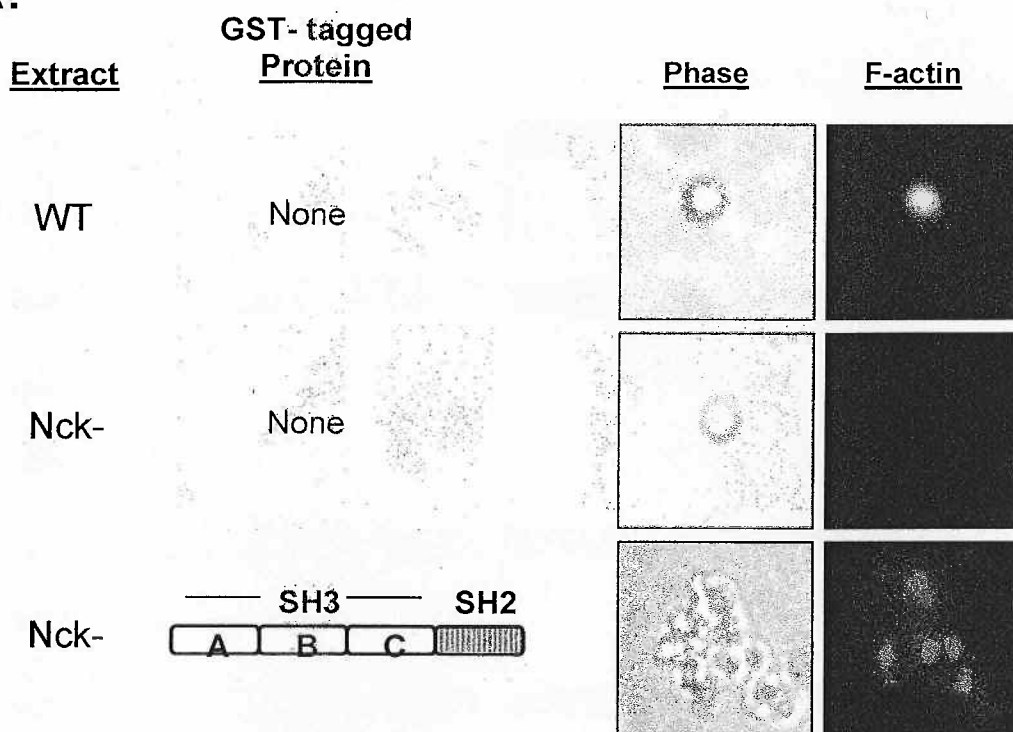


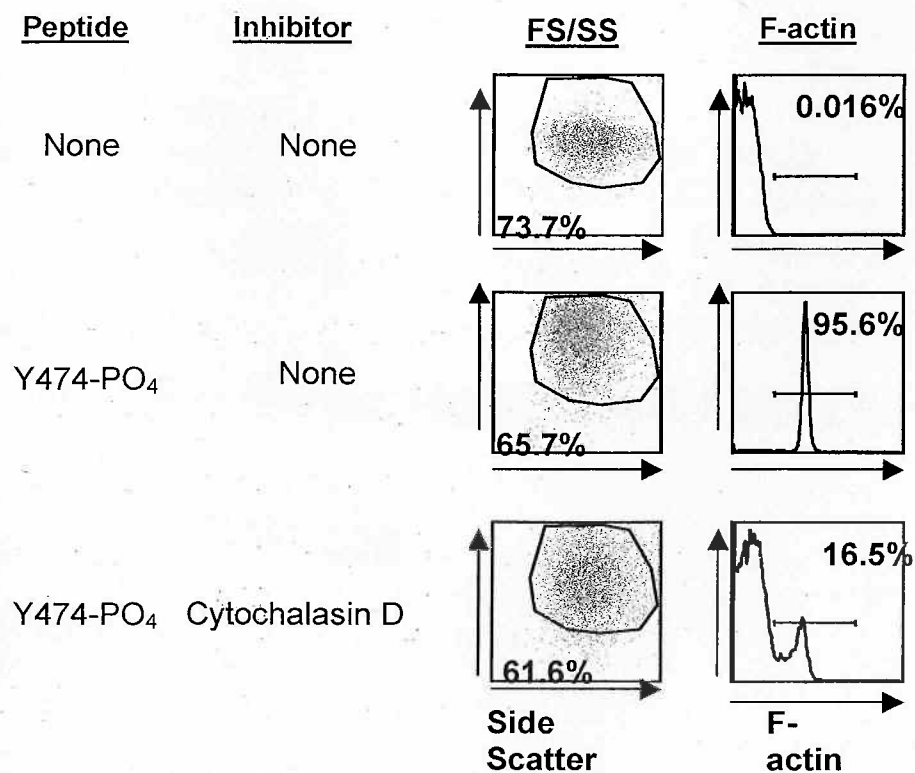
Figure 1. The central SH3 domain of Nck, SH3(B), is essential for maximal Tir-mediated pedestal formation on cultured fibroblasts

Plasmids containing the GFP-tagged Nck derivatives were transfected into Nck-null fibroblasts. Transfected monolayers were infected with wildtype EPEC and treated with DAPI to identify bacteria ('EPEC') and phalloidin ('F-actin') to visualize F-actin. The percentage of bound bacteria generating actin pedestals per positively transfected cell was scored. Additionally, pedestals were scored as 'robust', ie. intensely staining with phalloidin. Each experiment was comprised of 10 positively transfected cells. Experiments were performed at least 3 times. The white arrowhead indicates robust actin pedestal formation.

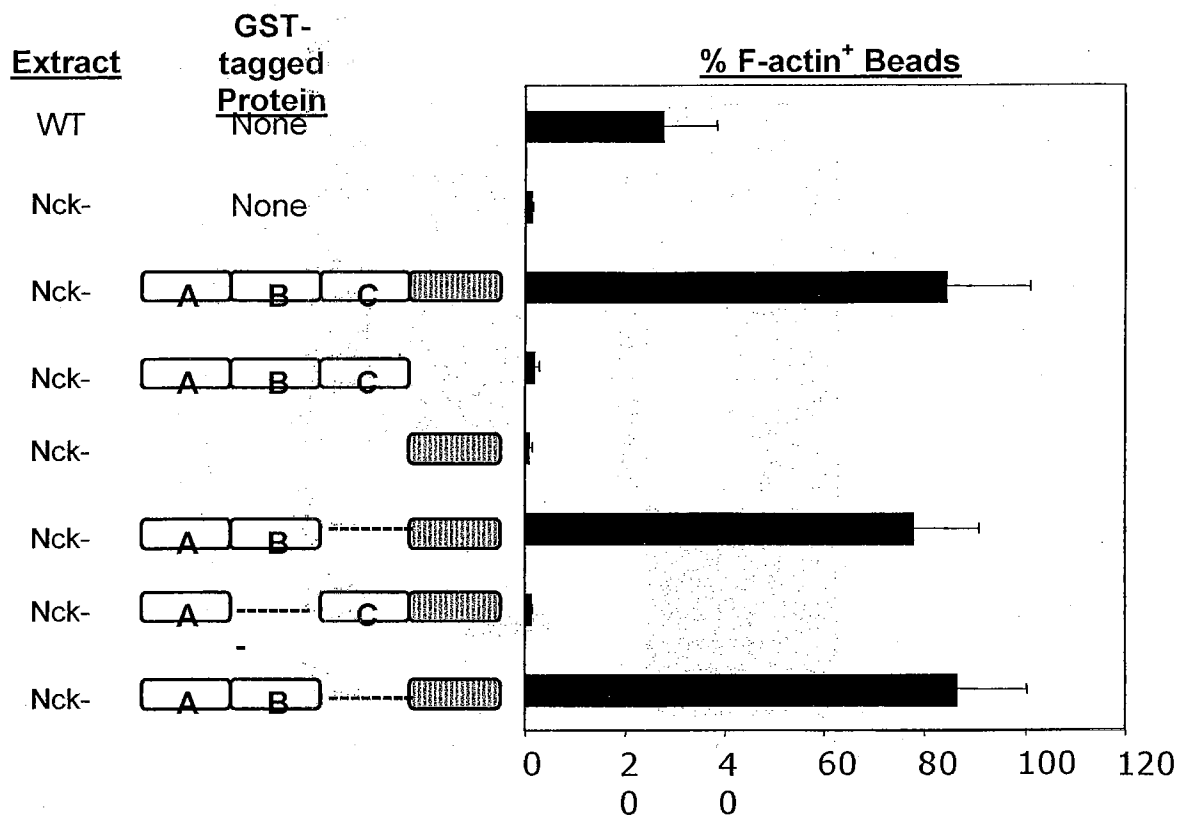
A.



B.



C.



D.

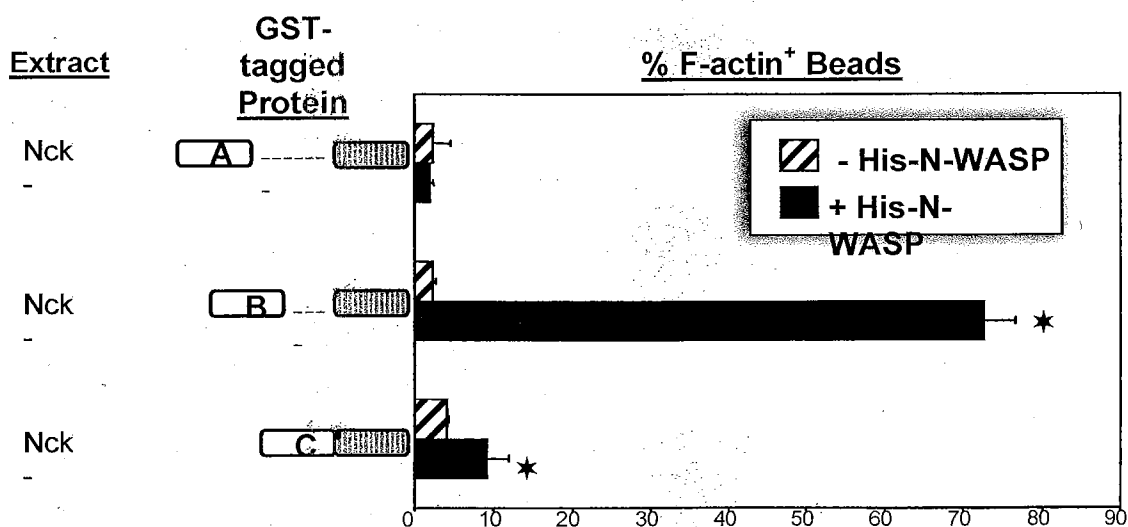


Figure 2. Recapitulation of Y474-dependent actin signaling in cell free extracts places emphasis on the SH2 and central SH3 domain of Nck for efficient actin accumulation.

(A). Wildtype and Nck-null fibroblasts were processed to generate cell-free extracts. In specified samples, Nck-null extracts were supplemented with 7nM recombinant Nck, treated with rhodamine-labeled actin and challenged with 1 μ M latex beads coated with a 12 amino acid Tir phosphopeptide. Actin assembly was examined using fluorescence microscopy under 6000x magnification.

(B). Nck-null extracts supplemented with 7nM GST-Nck treated with rhodamine-labeled actin and challenged with either uncoated latex beads or beads coated with the Y474 Tir phosphopeptide. In specified samples cytochalasin D (2 μ M) was added. Actin assembly was quantitated by flow cytometry and the percentage of F-actin positive beads determined.

(C). Nck-null extracts were supplemented with GST-tagged Nck derivatives (7nM) and treated as described above. Actin assembly was quantitated by flow cytometry and the percentage of F-actin positive beads determined. Data are the mean (\pm 95% CI) of 3 experiments.

(D). Nck-null extracts with supplemented with GST-tagged Nck derivatives (7nM) and His-tagged N-WASP (50nM) (black bars) or GST-tagged Nck derivatives only (gray bars). Extracts were treated as described above and actin assembly quantitated by flow cytometry. Data are the mean (\pm 95% CI) of 3 experiments, * $p \leq 0.01$ (Student's T-test).

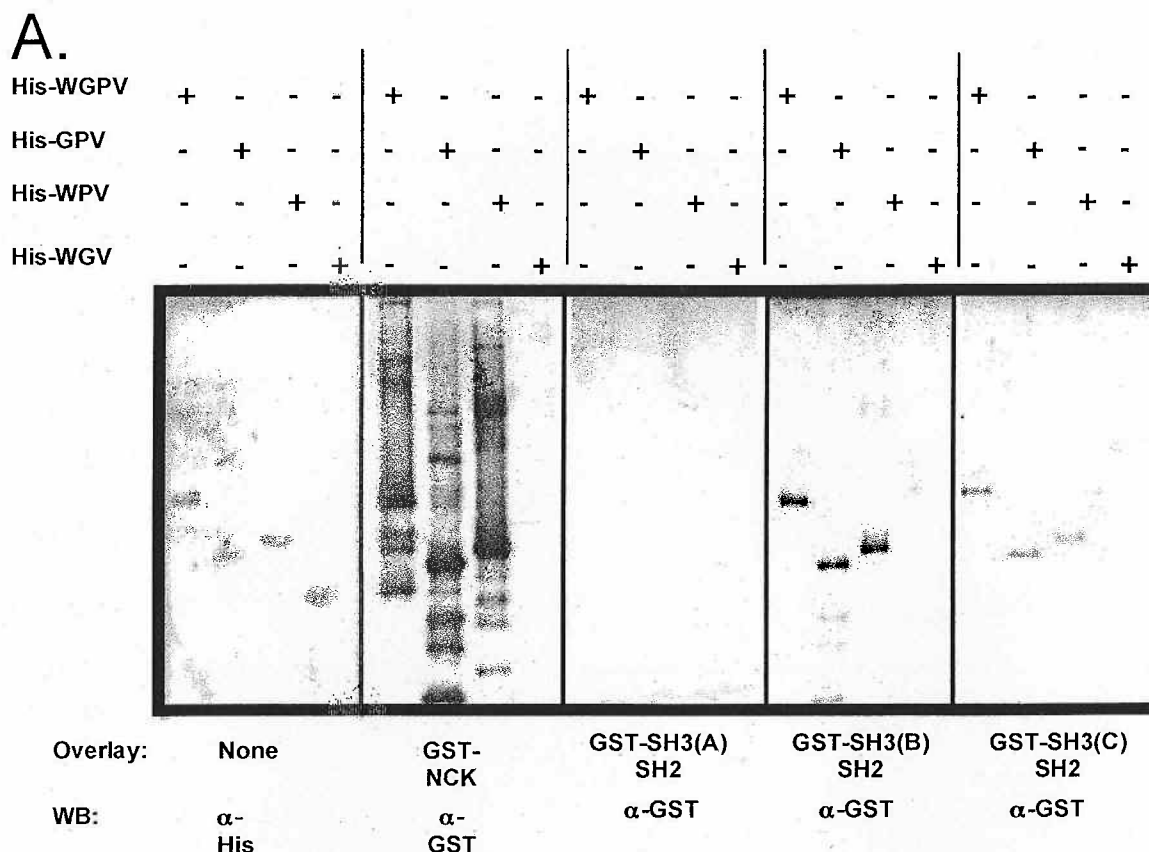


Figure 3. SH3(B)SH2 displays a higher affinity for recombinant N-WASP than other single SH3 domain Nck derivatives, but a lower affinity than full length Nck.

(A). Recombinant N-WASP derivatives were subjected to SDS-PAGE and Coomassie stained or transferred to nitrocellulose membrane and probed with GST-tagged Nck derivatives. Bound GST-tagged Nck derivatives were detected using goat anti-GST antibody.

(B) Microtiter wells were coated with His-tagged N-WASP 16nM) and probed with increasing concentrations of GST-tagged Nck derivatives (- \blacklozenge - Nck; - \blacksquare - SH3(A)SH2; - \blacktriangle - SH3(B)SH2; - \circ - SH3(C)SH2; - \triangle - SH2; - \bullet - GST only) and Nck binding was quantitated by ELISA using anti-GST antibody.

B.

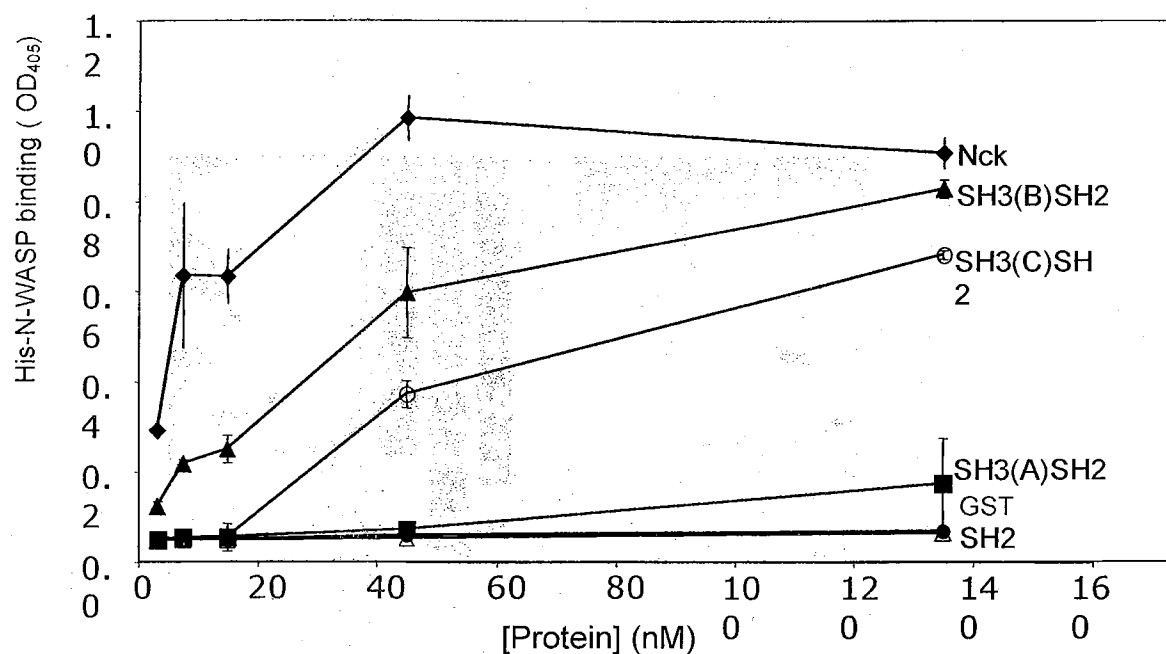


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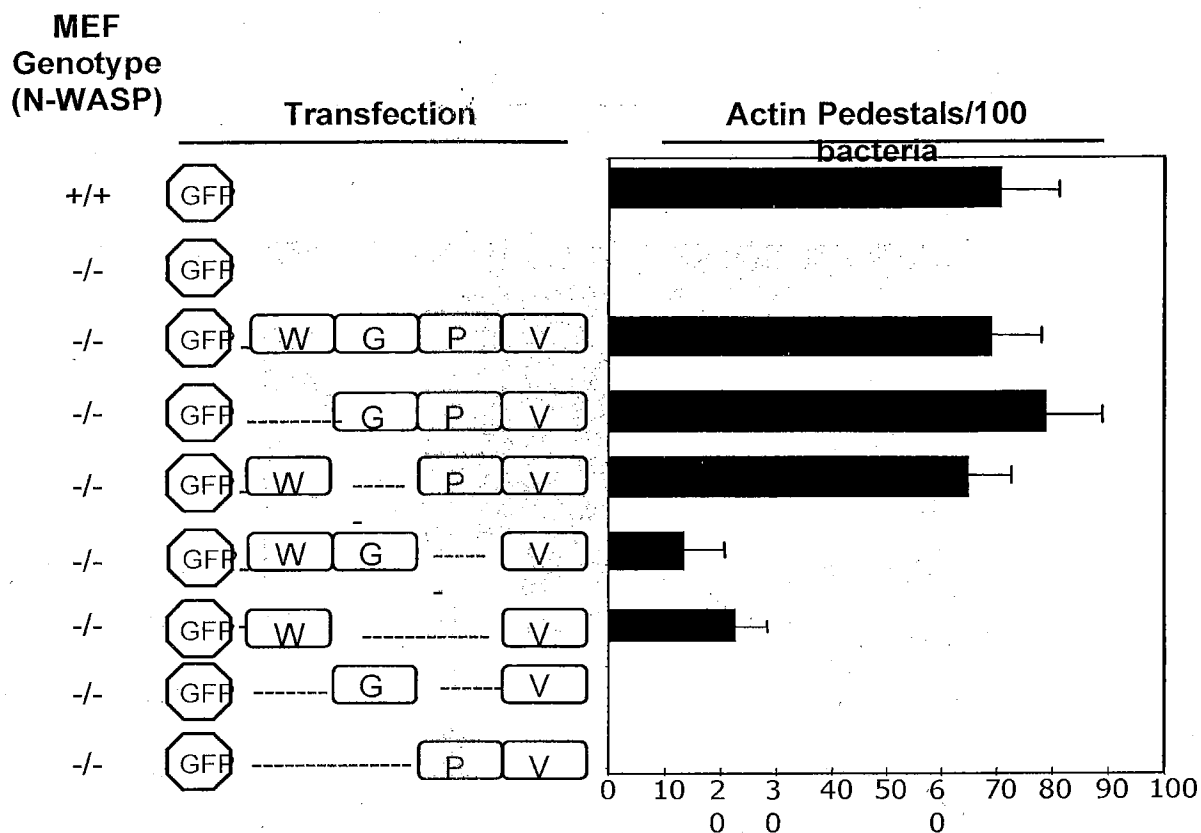


Figure 4. The WH1 domain of N-WASP is not required for wildtype levels of EPEC pedestal formation.

Wildtype MEFs (top row) or N-WASP-null MEFs (bottom rows) transfected with GFP-tagged N-WASP derivatives were infected with EPEC and treated with DAPI to identify bacteria and with phalloidin to visualize F-actin. To measure the efficiency of pedestal formation, the percentage of adherent bacteria associated with actin pedestals on cells expressing low levels of GFP-N-WASP was quantitated. Data are the mean (\pm 95% CI) of 3 experiments.

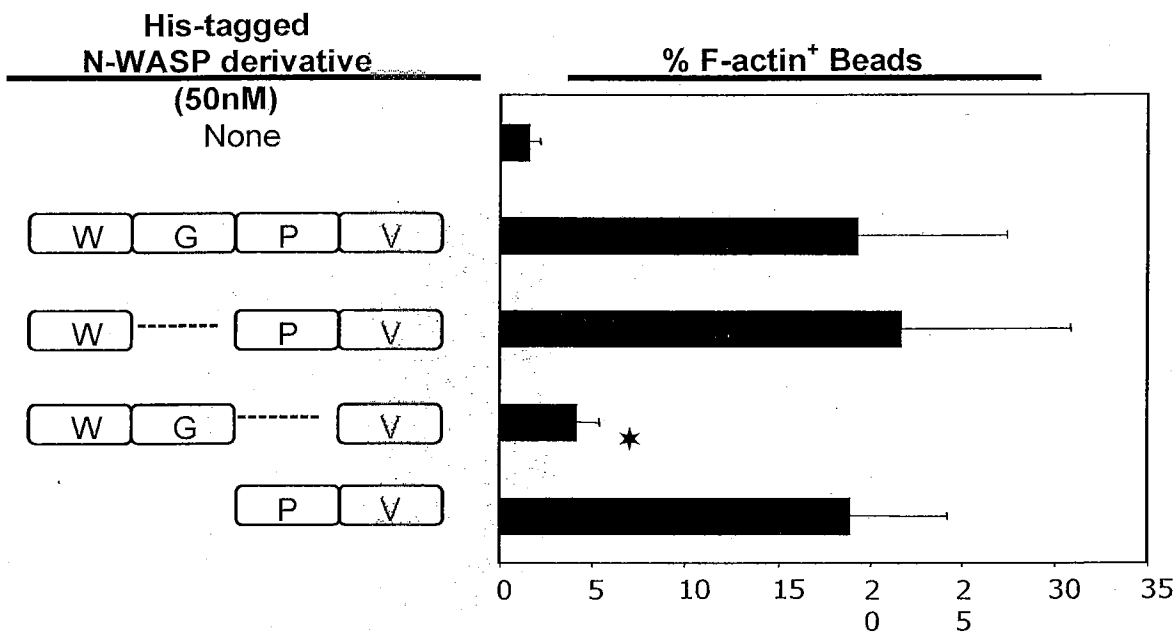


Figure 5. Only the PRD and the VCA domain of N-WASP are required for Tir-mediated signaling in N-WASP null extracts.

N-WASP-null fibroblasts were processed to generate concentrated cell free extracts. In specified samples, extracts were supplemented with select His-tagged N-WASP derivatives (50nM), treated with rhodamine actin and challenged with the Tir phosphopeptide-coated beads. Actin accumulation was quantitated by flow cytometry to determine the percentage of F-actin positive beads. Data are the mean (\pm 95% CI) of 3 experiments, $\star p \leq 0.02$ (Student's T-test).

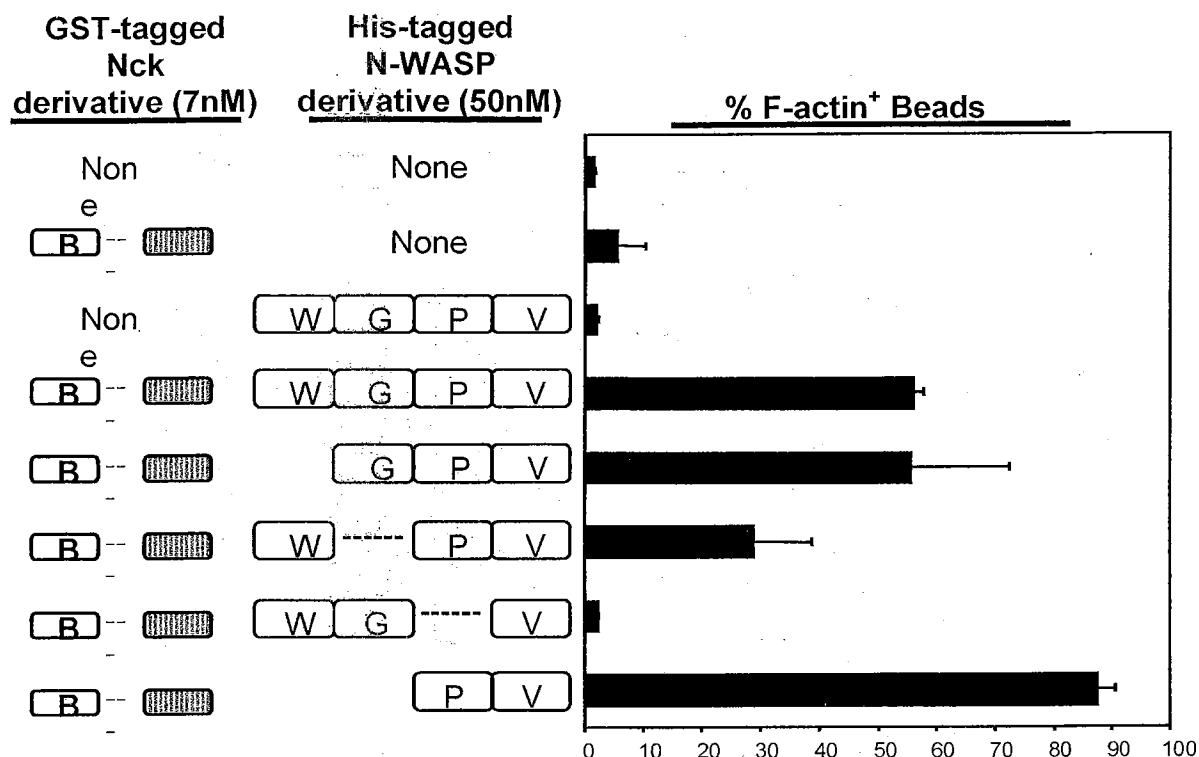
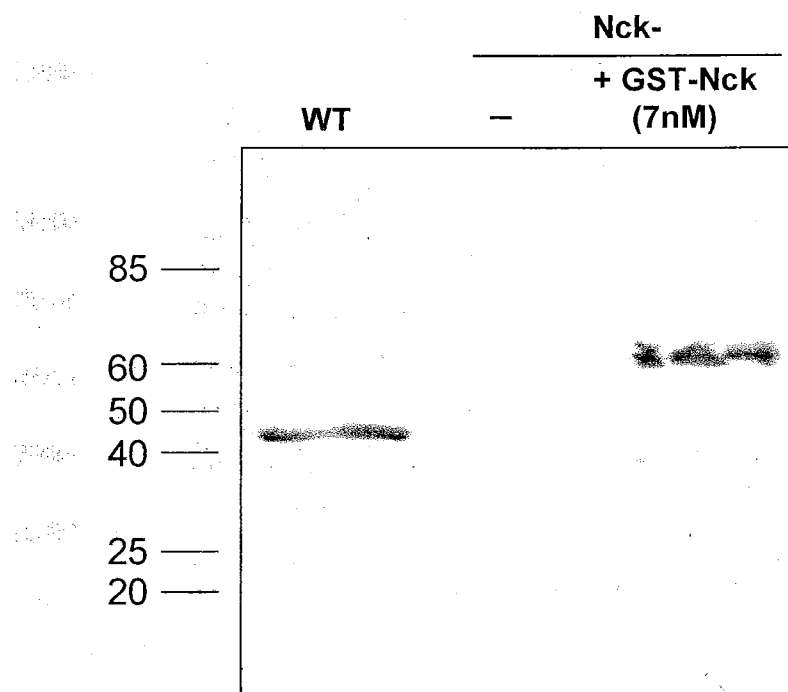


Figure 6. Only the PRD and the VCA domain of N-WASP are required for Tir-mediated, Nck-dependent signaling *in vitro*.

Nck-null fibroblasts were processed to generate cell free extracts and supplemented with GST-SH3(B)-SH3 (7nM) and His-tagged N-WASP derivatives (50nM) where indicated. Extracts were treated with rhodamine actin and challenged with Tir-phosphopeptide-coated beads. Actin accumulation was quantitated by flow cytometry to determine the percentage of F-actin positive beads. Data are the mean (\pm 95% CI) of 3 experiments.



Supplementary Figure 1. Endogenous levels of Nck in mammalian extracts are similar to those observed in GST-Nck supplemented Nck-null extracts.

Wildtype, Nck-null and GST-Nck (7nM) supplemented extracts were processed for SDS-PAGE, transferred to nitrocellulose membrane and probed with a polyclonal Nck antibody

CHAPTER III

INVESTIGATION OF NCK-INDEPENDENT ACTIN SIGNALING MEDIATED BY ENTEROPATHOGENIC *E. COLI*

Summary:

Our lab previously demonstrated that two Nck-independent actin signaling cascades are triggered by an EPEC infection. Though these additional pathways are not responsible for the majority of actin signaling, they do contribute to approximately twenty percent of actin pedestal formation. Understanding the eukaryotic requirements for these alternate pathways may not only give insight into actin pedestal formation triggered by a closely related pathogen, EHEC, but may also further elucidate the intricacies of general actin dynamics. The development of a Nck-null, N-WASP-silenced cell line has enabled us to examine the domain requirements of N-WASP for these alternate pathways, giving clues as to the identity of potential adaptor proteins required in these cascades. Furthermore, though initial work using Nck-null cell extracts indicated that the alternate pathways could not be detected, further technical refinement of the extract system has demonstrated that the Nck-independent pathways can be observed. Recapitulation of Nck-independent signaling using these extracts may facilitate identification of critical factors in this pathway.

Introduction

The most well-defined pathway for EPEC-mediated actin assembly involves recruitment of the eukaryotic adaptor protein, Nck, via the phosphorylation of a critical tyrosine at position 474 in the C-terminus of Tir (Kenny *et al.*, 1997). This phosphorylation event, mediated by a variety of host family kinases (Phillips *et al.*, 2004; Swimm *et al.*, 2004) Bommarius *et al.*, in press), results in the creation of a docking site for the SH2 domain of Nck, which directly recruits N-WASP to the membrane via the interaction between the second SH3 domain of Nck and the proline-rich region of N-WASP (Chapter 2). This entire recruitment process culminates with robust localized actin assembly beneath the sites of bacterial attachment in a pedestal-like structure.

However, this Nck-dependent signaling pathway is not solely responsible for EPEC-triggered actin assembly. In fact, EPEC is capable of stimulating actin pedestal formation on Nck-null fibroblasts albeit at a lower frequency than on wildtype cells (Campellone and Leong, 2005). Phillips *et al* demonstrated that a Y474F Tir mutant can still be phosphorylated *in vitro* and that tyrosine phosphorylation of Y474F Tir could be observed beneath adherent bacteria at low frequencies (Phillips *et al.*, 2004). Campellone *et al.* showed that infection of cells with the Y474F Tir mutant resulted in approximately 9% of bound bacteria associating with phosphotyrosine staining, and of that population, 30% generated actin pedestals. This pathway is independent of Nck, and an analysis of the C-terminus of EPEC Tir revealed an additional tyrosine, Y454, located twenty amino acids N-terminal to Y474, is phosphorylated. The Y454F/Y474F double mutant was unable to promote a significant level of phosphotyrosine staining and, subsequently, was unable to promote any actin pedestal assembly, indicating that a phosphorylated Y454

plays a significant role in EPEC-mediated Nck-independent signaling (Campellone and Leong, 2005).

EPEC is not the only pathogen to utilize multiple routes for actin assembly; the viral pathogen, vaccinia, stimulates two pathways to generate actin comet tails to facilitate intracellular movement (Frischknecht *et al.*, 1999). Vaccinia encodes a protein, A36R, containing two tyrosine residues twenty amino acids apart, to recruit eukaryotic factors to promote actin nucleation. The tyrosine residue responsible for the majority of the actin signaling, Y112, recruits Nck to the viral particle, resulting in the localization of numerous host factors, including WIP and N-WASP, culminating in actin assembly (Moreau *et al.*, 2000). A secondary pathway flows through Y132, which binds Grb2 via its SH2 domain thereby recruiting N-WASP (Frischknecht *et al.*, 1999; Scaplehorn *et al.*, 2002). However, it has been demonstrated that, although Grb2 is localized to the EPEC pedestal, the Grb2 SH2 domain is unable to bind to the phosphorylated forms of Y454 (or Y474) and overexpression of the Grb2 SH2 domain in cultured fibroblasts fails to inhibit EPEC triggered actin pedestal formation (Campellone and Leong, 2005).

In addition, a Nck-independent pathway that requires Y474 was also revealed. Approximately 25% of bacteria bound to Nck-null fibroblasts are still capable of generating actin pedestals. A Y474F Tir mutant generated pedestals on these cells at efficiencies approximately six- to ten-fold lower than did a wildtype EPEC strain, suggesting that approximately 10-15% of these pedestals is due to the Y454 pathway. By implication, approximately 85-90% of pedestals generated on Nck-null cells are likely mediated by residue Y474. Virtually nothing is known about how Y474 promotes Nck-independent actin pedestal formation, yet the Nck-independent signaling cascades

ultimately result in the recruitment of both N-WASP and the Arp2/3 complex, demonstrating that all three pathways converge upon N-WASP (Campellone and Leong, 2005).

The fact that multiple pathways, both Nck-dependent and -independent, appear to contribute to pedestal formation complicates its analysis. Analysis of N-WASP reported that the WH1 domain, rather than the GBD or the PRD, is responsible for mediating the recruitment of N-WASP to the sites of bacterial attachment (Lommel *et al.*, 2001). This suggests that additional host factors are required to mediate the interaction between Nck and N-WASP and that factor interacts with the WH1 domain. However, it is difficult to determine which EPEC-mediated signaling pathway is responsible for the WH1-dependent recruitment of N-WASP, because the wildtype EPEC strain used in the studies stimulates all three actin signaling cascades.

In fact, quantitation of pedestal formation promoted by different deletion derivatives of N-WASP is consistent with the hypothesis that a Nck-independent, rather than the Nck-dependent, pathway utilizes the WH1 domain of N-WASP to promote pedestals. Our previous analysis of the domain requirements of N-WASP for EPEC-triggered actin pedestal formation revealed that ectopic expression of an N-WASP derivative comprised solely of the WH1 domain and the VCA domain in N-WASP null fibroblasts was capable of stimulating a low, yet significant, level of EPEC pedestal formation. The percentage of bound EPEC generating actin pedestals on these cells was approximately 25%, a level exceeding that observed on Nck-null cells infected with the same bacteria (Campellone and Leong, 2005). Based on this observation, as well as the finding that the only domains of N-WASP required for Nck-dependent signaling are the

PRD and VCA, one may postulate that the required host factors for the Nck-independent signaling pathways interact with the WH1 domain of N-WASP. In the current study, we have determined which domains of N-WASP promote pedestal formation on Nck-null cells.

Materials and Methods

Generation of Nck-null, N-WASP silenced cell line

Nck-null mouse embryonic fibroblasts were grown in six-well plates until ~90% confluence and transfected with 1mg of N-WASP shRNA plasmid (Open Biosystems) per well for 12 hours using Lipofectamine Plus reagent (Invitrogen). Cells were washed with PBS and grown under puromycin selection (5 μ g/mL) until confluency, when they were reseeded into a T-25 culture flask and grown under higher concentrations puromycin (10 μ g/mL). The knockdown of N-WASP was confirmed by western blotting using a polyclonal N-WASP antibody (gift from the Kirschner lab) and loading volume confirmed with α -tubulin antibody (1:500, Molecular Probes).

Transfections and Infections

Nck-null, N-WASP-silenced mouse embryonic fibroblasts were grown in six-well plates until ~90% confluence and transfected with 500ng of GFP-tagged plasmid per well for 12 hours using Lipofectamine Plus reagent (Invitrogen). Cells were reseeded onto 12-mm glass coverslips to achieve 50-75% confluency after an additional 24 hours of growth at 37°C in a 5% CO₂ atmosphere. Cells were infected with 2 x 10⁶ EPEC or the Y454F EPEC Tir mutant in DMEM+3% FBS + 25mM HEPES pH 7.4 for 3.5 hours at 37°C in a 5% CO₂ atmosphere before being fixed and permeablized for fluorescence microscopy.

Fluorescence Microscopy

Infected monolayers were fixed in PBS + 2.5% paraformaldehyde for 30 min and permeabilized with PBS + 0.1% Triton X-100 for 5 min. Bacteria were visualized by treatment with DAPI (1 μ g/mL; Sigma Aldrich) and F-actin was identified with TRITC-phalloidin (1 μ g/mL; Sigma Aldrich). Positively transfected cells were identified by GFP expression. Pedestal formation efficiency was quantitated by identifying positively transfected cells with at least five EPEC bound, scoring the number of pedestals formed and calculating the percent of bound bacteria generating pedestals. Ten positively transfected cells meeting the above requirements were examined per experiment and each experiment was repeated at least three times.

Generation of cell free extracts

Nck-null and N-WASP null mouse embryonic fibroblasts were grown in tissue culture flasks with surface areas of 636 cm² (Corning) until they reached between 70 and 90% confluence. The culture medium was removed and cells were washed with sterile 1X PBS (137mM NaCl, 8mM sodium phosphate, 1.5mM potassium phosphate, 2.7mM KCl, pH 7.4). Cells were trypsinized at 37°C and collected by centrifugation (10 min, 37°C, 300g), the pellets resuspended in PBS and recentrifuged (10 min, 37°C, 300g). The pellet was resuspended in 10mL of permeabilization buffer (80mM Pipes-KOH, pH 6.75, 1mM MgCl₂, 1mM EGTA, 0.5% Triton X-100 (vol/vol) and 10% glycerol (vol/vol) supplemented with 5mM CaCl₂). These cells were incubated for 10 minutes at 4°C, with gentle agitation. Post incubation, additional EGTA was added to a final concentration of 10mM. The extracts were centrifuged (20min, 4°C, 200,000g) in an 80ti rotor for the Beckman Coulter Optima L-100 XP Ultracentrifuge. The supernatant was divided into

50 μ L aliquots, snap frozen in liquid nitrogen and stored at -80°C until further use. To generate concentrated extracts, 1mL aliquot were subjected to centrifugation in a Microcon Centrifugal Filter Unit (MW cut off 10kD, Millipore) for 30' at 14,000 rpm at 4°C .

Pulldowns and Western Blotting

One hundred microliters of extracts (unconcentrated or concentrated) were thawed on ice and incubated with recombinant proteins (where indicated) for at least 1 hour on ice. Peptide coated magnetic beads (Invitrogen) were added to the extracts and incubated for 30 minutes at 25°C . Beads were recovered using a magnet and washed three times in PBS + 0.05% Tween-20, 10' at 25°C with gentle agitation. Beads were resuspended in 2x SDS-PAGE loading buffer and processed for Western blotting using a polyclonal N-WASP antibody (1:2000, gift from the Kirschner lab).

Results

The WH1 domain is required for Nck-independent actin pedestal formation mediated by Tir Y474

Given the complexity of EPEC actin signaling conferred by the multiple routes to pedestal formation, it is possible that the residual actin pedestal formation observed on N-WASP-null fibroblasts complemented with an N-WASP derivative comprised of either the WH1 and the VCA domains ('WV') or the WH1, GTPase binding and VCA domains ('WGV') is due to the Nck-independent signaling cascades. To investigate the contribution of different domains of N-WASP to the Nck-independent signaling cascades, we created a Nck-null, N-WASP-silenced fibroblast cell line by transfecting

five different commercially available shRNA plasmids targeting N-WASP into the Nck-null mouse embryonic fibroblasts used in previous studies (Figure 1, table for shRNA sequences). Western blotting of the five N-WASP-silenced cell lines, the parental Nck-null fibroblasts and N-WASP-null fibroblasts with a polyclonal N-WASP antibody revealed a dramatic decrease in N-WASP proteins levels in the five silenced cell lines as compared to the Nck-null parental cell line. Cell lines #4 and #5 appeared to have the greatest reduction in N-WASP protein levels (Figure 1, bottom) and were evaluated for their ability to support pedestal formation by wildtype EPEC. Nck-null, N-WASP-silenced cell line #4 had the greatest reduction in pedestal formation and was used for the following studies.

Infection of the Nck-null, N-WASP silenced fibroblasts with wildtype EPEC demonstrated that the silencing of N-WASP was effective. Only 2% of bound EPEC generated pedestals on the Nck-null, N-WASP-silenced cell line as compared to the 13% of bound bacteria capable of accumulating actin on the parental Nck-null cell line. However, the potential for off-target silencing events that could globally impair the cell's ability to focus actin exists, therefore we infected the same cell line with an EPEC mutant possessing EHEC Tir on the bacterial chromosome and a plasmid bearing the critical EHEC effector molecule, EspFu. Since EHEC (and this engineered EPEC strain) have the ability to stimulate actin pedestal formation independently of Nck and N-WASP, infection of the Nck-null, N-WASP-silenced fibroblasts with this mutant EPEC strain could determine if there were any off-target silencing events that could impair actin assembly. However, the Nck-null, N-WASP-silenced fibroblasts were capable of supporting pedestal formation by this EPEC mutant. Ectopic expression of GFP-tagged

N-WASP in the Nck-null, N-WASP-silenced fibroblasts promoted actin accumulation beneath approximately 21% of adherent bacteria (Figure 2, black bars). Transient expression of N-WASP derivatives comprised of the GTPase binding and VCA domains ('GV') or the proline-rich and VCA domains ('PV') were incapable of promoting any actin accumulation beneath the sites of adherent wildtype EPEC (Figure 2, black bars), a result similar to that observed on N-WASP-null fibroblasts transfected with the same derivatives and challenged with wild type EPEC (Chapter II, Figure 6). However, ectopic expression of an N-WASP derivative comprised of the WH1 and VCA domains ('WV') restored wild type EPEC pedestal formation to ~23% of bound bacteria accumulating actin, a percentage statistically indistinguishable from that observed on fibroblasts transfected with full-length N-WASP (Figure 2, black bars). The ability of the WV derivative to restore wild type EPEC pedestal formation to a level similar to that observed with full length N-WASP indicates that the WH1 domain plays a key role in transducing the signals required for the Nck-independent actin assembly cascade.

To investigate the domains of N-WASP specifically required for the Y474-dependent, Nck-independent actin signaling cascade, we performed a similar set of experiments with an EPEC Tir mutant carrying a tyrosine to phenylalanine amino acid change at position 454 (Y454F Tir mutant), which effectively disables the Y454-dependent, Nck-independent arm of EPEC pedestal formation. Infection of the Nck-null, N-WASP-silenced fibroblasts with the Y454F Tir mutant did not result in any actin pedestal formation, while infection of fibroblasts transfected with full-length N-WASP with this Tir mutant resulted in approximately 14% of bound bacteria accumulating actin beneath the sites of attachment (Figure 2, gray bars). Furthermore, expression of N-

WASP derivatives encompassing the GTPase binding and VCA domains ('GV') or the proline-rich and VCA domains ('PV') could not promote pedestal formation by the Y454F Tir mutant on the Nck-null, N-WASP-silenced fibroblasts, a result consistent with that observed in wild type EPEC infections of similarly transfected fibroblasts (Figure 2). Transient expression of an N-WASP derivative comprised of the WH1 and VCA domains ('WV') restored pedestal formation to 14% of adherent bacteria, an efficiency indistinguishable from that observed on the fibroblasts expressing full length N-WASP (Figure 2, gray bars).

Although the observation that the Y454F Tir mutant generated pedestals on cells transfected with either full length N-WASP or WV less efficiently than wild type EPEC is consistent with the hypothesis that Y454 contributes to the Nck-independent signaling cascades, it is difficult to directly compare the strains because they are not isogenic. The Y454F Tir mutant carries a plasmid containing HA-tagged Tir, which has exhibited moderately lower translocation levels presumably due to the HA tag, (K. Campellone, unpublished observations). To directly compare levels of pedestal formation stimulated by each strain, an EPEC Tir mutant carrying full length HA-tagged Tir should be employed. Nevertheless, these initial experiments reveal that an N-WASP derivative comprised of the WH1 and VCA domains is sufficient to function in the Y474-dependent, Nck-independent signaling cascade, potentially providing clues as to possible candidate molecules involved in this pathway.

Attempts to recapitulate Tir-mediated, Nck-independent actin assembly in mammalian cell-free extracts

An attempt to visually detect actin accumulation around Tir phosphopeptide-coated beads in concentrated Nck-null fibroblast extracts gave equivocal results (data not shown), but raised the possibility that host components might be recruited to the beads, albeit at a lower efficiency than that observed in Nck-proficient extracts. To examine if critical factors, such as N-WASP, were recruited to the beads, we challenged unconcentrated and concentrated Nck-null fibroblast extracts with magnetic beads coated with either the Y474 phosphopeptide or, as a negative control, the Y474F peptide. Additionally, to test whether this assay system could detect the known interaction between N-WASP, Nck and the Y474 peptide, the same beads were added to unconcentrated extracts supplemented with 7nM GST-Nck. The beads were recovered from all extract samples and processed for Western blotting with a polyclonal N-WASP antibody. In the unconcentrated extracts supplemented with GST-Nck, endogenous N-WASP could be detected on Y474 phosphopeptide-coated beads but not on the Y474F peptide coated beads, indicating that the Y474 phosphopeptide-coated beads are able to recruit N-WASP (Figure 3, left panel). As expected, endogenous N-WASP could not be detected in either the concentrated and unconcentrated extracts challenged with the Y474F peptide coated beads (Figure 3, both panels). Beads coated with the Y474 phosphopeptide were unable to recover N-WASP from either the concentrated or unconcentrated Nck-null extracts (Figure 3, right panel). Given previous results where actin assembly could be restored by the addition of recombinant N-WASP to Nck-null extracts supplemented with GST-SH3(B)SH2, a Nck derivative previously unable to promote actin assembly (Chapter 2, Figure 2C), it would be reasonable to postulate that the addition on recombinant N-WASP to the Nck-null extracts could boost the signal to detectable levels, but this hypothesis is untested.

Discussion

Initial studies of EPEC-mediated actin assembly resulted in a model in which phosphorylated Tir Y474 triggered a Nck-dependent signaling cascade leading to the formation of actin pedestals beneath sites of bacterial attachment. However, as investigation has continued, it has become apparent that rather than stimulating actin assembly via a single peptide sequence and a single adaptor, EPEC utilizes two phosphotyrosine-containing sequences to stimulate at least three pathways: a primary pathway that requires Nck and two secondary pathways that utilize unknown factors to recruit N-WASP (Campellone and Leong, 2005). Therefore, the previous assessment that specific domains of N-WASP, such as the WH1 domain (Lommel *et al.*, 2001), are required for pedestal formation, may not apply specifically to the Nck-dependent pathway. Our discovery that the PRD and the VCA are the only two domains of N-WASP required for Tir-mediated, Nck-dependent actin pedestal formation (Chapter 2) suggested that the requirement for the WH1 domain may instead be for one or both of the Nck-independent pathways.

The development of a Nck-null, N-WASP-silenced cell line facilitated the evaluation of domain requirements of N-WASP for the Nck-independent pathways. We found that the knockdown of N-WASP in the cell line reduced the pedestal formation from approximately 15% of bound bacteria generated pedestals to 2%, a reduction significant enough to enable an evaluation of the N-WASP domains. However, one must account for the potential of the remaining low levels of N-WASP in these silenced cells to stimulate actin assembly. To determine if the remaining endogenous N-WASP is truly

capable of supporting Nck-independent actin pedestal formation, further evaluation of pedestal formation with an EPEC strain lacking Tir or the Y454F/Y474F Tir mutant should be conducted to determine the absolute level of background associated with these cells. Furthermore, the development of a clonal population of the Nck-null N-WASP-silenced fibroblasts completely impaired for EPEC pedestal formation would eliminate any potential assistance from endogenous N-WASP.

Expression of full length N-WASP in these cells restored pedestal formation to 21% of bound bacteria generating pedestals, a level statistically indistinguishable from that observed on Nck-null fibroblasts, where approximately 13% of bound EPEC formed pedestals. The expression of GBD-VCA (GV) or PRD-VCA (PV) did not restore pedestal formation, but expression of WH1-VCA (WV) in these cells supported a high level of Nck-independent actin assembly, with 23% of bound bacteria generating pedestals. Interestingly, WH1-VCA was capable of promoting a similar level of EPEC pedestal formation when expressed in Nck-proficient, N-WASP-null fibroblasts (Chapter 2, Figure 4). This result supports the hypotheses that either Nck-deficiency or removal of the PRD each reveals one or more Nck-independent pathways, which together promote pedestal formation at approximately one-quarter wild type efficiency, and that an intact PRD is essential for the Nck-dependent pathway of pedestal formation.

Similar results were obtained when the cells were challenged with EPEC expressing Y454F Tir, implying that the WH1 domain is critical for Nck-independent signaling via Tir Y474. Identification of the domain(s) of N-WASP essential for signaling via Tir Y454 using a similar approach is technically difficult due to the relatively low level of pedestal formation—approximately 5% of wild type—stimulated

by that pathway. Presumably, in the absence of Nck, Tir Y474 facilitates the formation of an actin-signaling complex in which N-WASP is recruited (and perhaps activated) via the WH1 domain, suggesting that Tir Y474 might directly bind to a host factor that interacts with the WH1. Once again, WIP or WIP-like proteins, such as CR-16, WICH and WIRE, arise as contenders for a role in this signaling cascade. WIP plays an integral part in the generation of vaccinia actin comet tails by recruiting N-WASP to the virus to promote Nck-dependent signaling (Moreau *et al.*, 2000). It has been shown that the majority of N-WASP exists in a complex with WIP (Ho *et al.*, 2004) via an unusual interaction where WIP interacts with the WH1 domain (Peterson *et al.*, 2007; Volkman *et al.*, 2002). Upon activation of N-WASP, WIP is removed from the WH1 domain by another eukaryotic factor, Toca-1 (Ho *et al.*, 2004), implying that WIP can function as both a positive and negative regulator of N-WASP activity. It has been demonstrated previously that EPEC can generate pedestals on WIP-null cell lines (S. Snapper, unpublished data), which is not surprising given the result that the dominant actin signaling cascade triggered by EPEC requires only a direct interaction between Nck and N-WASP, therefore masking any subtle defect that may be conferred by a loss of WIP. To address the role of WIP in the Nck-independent signaling cascades, a variety of approaches could be used. First, one could silence WIP in Nck-null fibroblasts with commercially available shRNA constructs and assess whether EPEC requires this protein for pedestal formation. However, the commercially available shRNAs may not silence the WIP-like proteins that could potentially function in the absence of WIP. Therefore, one could complement the Nck-null N-WASP-silenced fibroblasts with an N-WASP derivative possessing a point mutant in the WH1 domain predicted to disrupt the WIP interaction. The majority of point

mutants in patients with Wiskott-Aldrich syndrome are located in the WH1 domain of WASP (Zetl and Way, 2002), where the most frequently isolated mutated residue is arginine 76 (Imai *et al.*, 1999; Miki *et al.*, 1996; Moreau *et al.*, 2000). By changing this residue to cysteine, histidine, leucine or proline (all which have been isolated from Wiskott-Aldrich syndrome patients), one could disrupt the N-WASP-WIP interaction. Transfection of the Nck-null N-WASP-silenced cell lines with an N-WASP derivative possessing this point mutation may address whether WIP or WIP-like proteins are involved in the Nck-independent signaling cascades.

The identification of the WH1 as critical to Nck-independent pedestal formation would also be helpful in an unbiased search for the unknown adaptor(s). For example, it might be possible to pull down the putative adaptor from Nck-null extracts using beads coated with Tir Y474 phosphopeptide. We have been able to successfully recover endogenous N-WASP from Nck-null extracts supplemented with recombinant Nck in a phosphorylated Y474-dependent fashion, but attempts to reproduce Tir-mediated Nck-independent signaling in extracts has been unsuccessful so far (Figure 3). If one could reproduce actin assembly in extracts from Nck-null fibroblasts using Tir Y474 phosphopeptide, one would be assured that all of the essential components were present, and analysis of the proteins on the beads by SDS-PAGE and mass spectrometry could provide a list of candidates for the Tir-interacting factor. Inclusion of a control assay using beads coated with the Y474F Tir peptide may allow for the identification of proteins that bind the beads in a non-specific manner. Once the beads are recovered from the extracts and processed, Far Western assays using recombinant His-tagged WH1 domain can be performed to identify the proteins recovered from these extracts that are

capable of interacting with the WH1 domain, thereby narrowing the number of candidates. One complication of such an approach is that the beads would be predicted to be associated with F-actin, and thus also with the myriad of F-actin-binding proteins present in cell extracts. A potential approach to eliminating these proteins from the beads is to preclude the stimulation of the actin signaling cascade by including recombinant N-WASP lacking the C-terminal VCA region in the extract, effectively stopping the signaling cascade from going to completion while allowing the necessary adaptors to bind to the phosphopeptide. However, in the Tir phosphopeptide studies presented here, Nck-independent actin assembly or recruitment of N-WASP was not observed, indicating that further refinement of the extract system, perhaps by modifying the preparation of the extracts and/or supplementing them with recombinant N-WASP derivatives, must occur to expedite the search for the putative (non-Nck) adaptor that links Tir Y474 to N-WASP.

Finally, it is possible, indeed likely, that several host molecules in addition to the putative adaptor and N-WASP function to promote actin pedestal formation. Although a WH1-VCA N-WASP derivative is sufficient to complement N-WASP for Nck-independent signaling pathway, other domains may play a role in the process by providing another layer of regulation to this process. For example, since the WH1-VCA construct lacks the GBD, a region of N-WASP required for autoinhibition, this derivative presumably requires no signals to disrupt this autoinhibition. To address this concern, further evaluation of additional N-WASP constructs that include the GBD should be performed. Nevertheless, the discovery of additional signaling cascades stimulated by EPEC has redirected the manner in which we examine actin pedestal formation mediated by this pathogen. By taking advantage of this knowledge as well as the new tools

developed here, we can critically examine which signaling components are involved in each pathway, thereby simplifying what is a very complex process.

<u>Oligo ID</u>	<u>Sequence</u>
TRCN0000099640 (1)	CCGGAGATTAACCAAGGCAGATATTCTCGAGAATATCTGCCTT GGTTAATCTTTTTTG
TRCN0000099641 (2)	CCGGGCTGGAGATACTTGTCAAGTACTCGAGTACTTGACAAGT ATCTCCAGCTTTTTG
TRCN0000099642 (3)	CCGGGCTTGTCTGGTTAAGGACAATCTCGAGATTGTCCTTAAC CAGACAAGCTTTTTG
TRCN0000099643 (4)	CCGGAGATTAACCAAGGCAGATATTCTCGAGAATATCTGCCTT GGTTAATCTTTTTTG
TRCN0000099644 (5)	CCGGTCAGGAAACAAAGCAGCTCTTCTCGAGAAGAGCTGCTTT GTTTCCTGATTTTTG

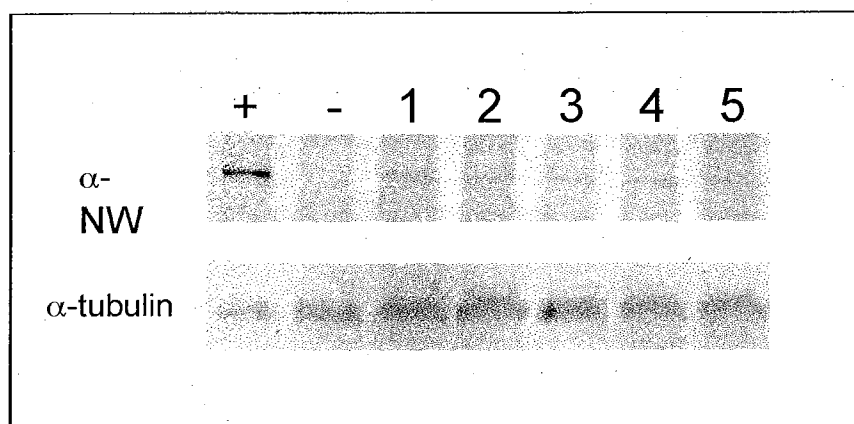


Figure 1. Generation of Nck-null N-WASP-silenced fibroblasts.

Nck-null fibroblasts were transfected with 1mg/mL of a series of N-WASP shRNA constructs (see table). Resulting cell lines were evaluated by Western blotting to evaluate the reduction in N-WASP using a polyclonal antibody. Western blotting with anti-tubulin antibody was included for a loading control.

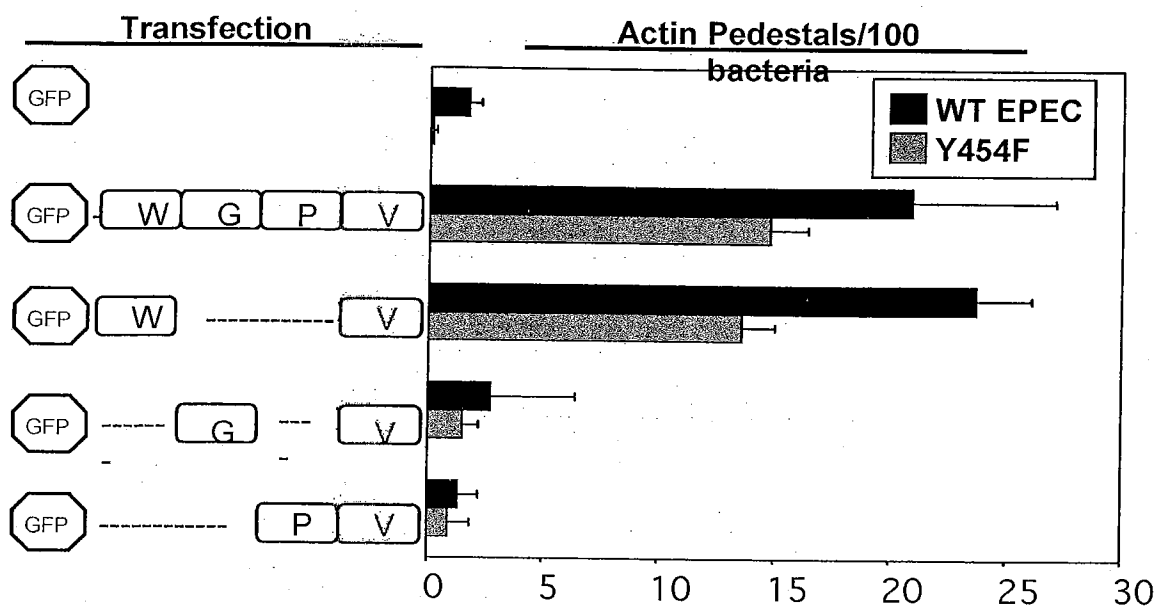


Figure 2. The WH1 and VCA domains of N-WASP are sufficient for Y474-dependent, Nck-independent actin pedestal formation.

Nck-null, N-WASP-silenced fibroblasts were transfected with GFP-tagged N-WASP derivatives, infected with either wildtype EPEC or a Y454F Tir mutant, treated with DAPI to identify bacteria with phalloidin to visualize F-actin. To measure the efficiency of pedestal formation, the percentage of adherent bacteria associated with actin pedestals on cells expressing low levels of transfected N-WASP construct was quantitated. Data are the mean (\pm 95% CI) of 3 experiments

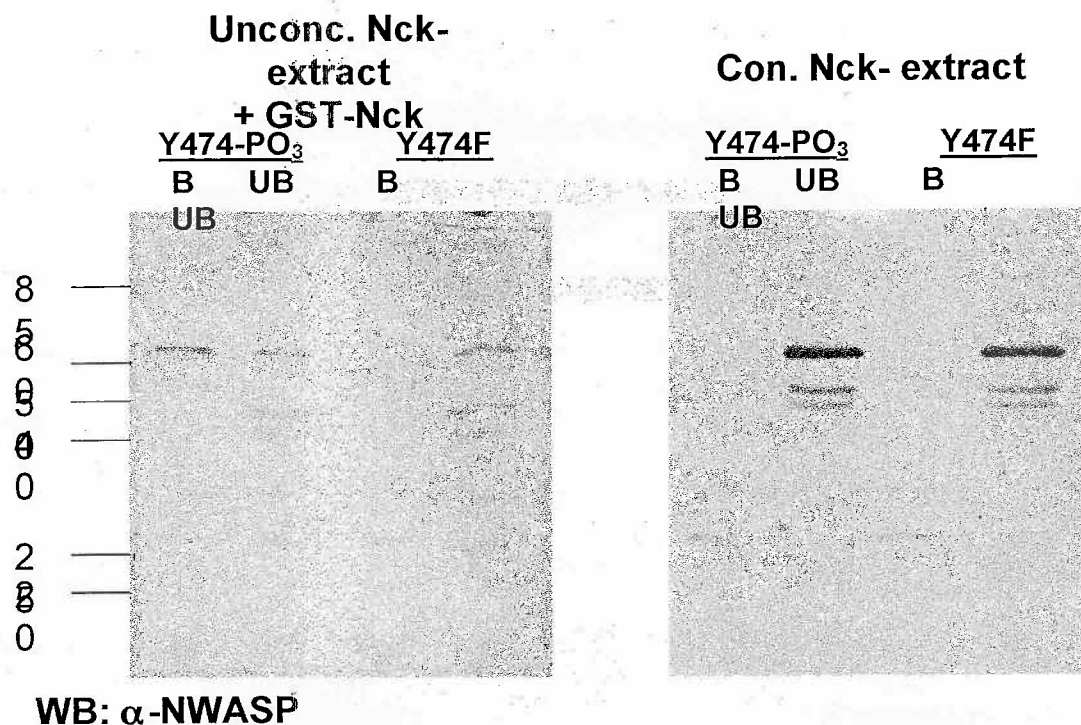


Figure 3. Tir phosphopeptide coated beads can recover N-WASP from unconcentrated, Nck-supplemented extracts but are unable to recover endogenous N-WASP from concentrated Nck-null extracts.

Unconcentrated Nck-null extracts were supplemented with 7nM GST-Nck and incubated with magnetic beads coated with either the Tir Y474 phosphopeptide or the Y474F peptide (left panel). Concentrated extracts were prepared in a similar fashion (right panel). Beads were recovered, prepared for SDS-PAGE and blotted for N-WASP using a polyclonal N-WASP antibody.

CHAPTER IV

DISCUSSION

The Nck-dependent pathway: A direct route to pedestal formation

Investigations of EPEC actin pedestal formation prior to the identification of the additional Nck-independent signaling cascades resulted in complex models that postulated additional eukaryotic factors to promote interaction of Nck and N-WASP and recruitment of the Arp2/3 complex. Though Nck can directly activate N-WASP *in vitro*, this interaction did not appear to stimulate the activity of N-WASP to generate actin pedestals, because the proline rich domain of N-WASP, i.e., the region where Nck is known to bind, was found to be dispensable for EPEC pedestal formation. Instead, an N-WASP derivative lacking the WH1 domain was impaired for EPEC pedestal formation. Interestingly, vaccinia, which also recruits Nck, requires the WH1 domain for proper targeting of N-WASP to the virus (Moreau *et al.*, 2000). This recruitment requires an additional protein, WIP, known to bind to the WH1 domain, because actin assembly and N-WASP recruitment were blocked by overexpression of a dominant negative form of WIP, and by mutations in the WH1 domain predicted to disrupt the WIP-N-WASP interaction. Subsequently, it would be reasonable to hypothesize that EPEC behaved in a similar fashion to vaccinia. Yet when similar experiments were conducted to test the requirement for WIP in EPEC pedestal formation, it was determined that the same set of mutations in the WH1 domain of N-WASP had no effect on the recruitment of N-WASP to the sites of bacterial attachment nor did they impair actin assembly (Lommel *et al.*, 2004). Additionally, EPEC could generate pedestals on WIP-null fibroblasts (S. Snapper,

unpublished data). Therefore, it would appear that WIP may not have a role in mediating the interaction between Nck and N-WASP during EPEC pedestal formation. It is possible that one of the multiple WIP-like proteins provide similar function in promoting interactions between Nck and N-WASP. Alternatively, the discovery of additional Nck-independent signaling pathways raised the possibility that the apparent requirement for the WH1 domain in pedestal formation was due to a role in Nck-independent signaling. Therefore, we embarked on a detailed analysis of Nck and N-WASP domains required for the Y474-dependent, Nck-dependent signaling cascade.

Our analysis revealed that the SH2 domain and the second SH3 domain of Nck were sufficient for robust Tir-mediated actin assembly. Additionally, our re-evaluation of the N-WASP domains required for general EPEC-mediated actin assembly culminated in a much different result than previous studies. Our investigation not only included a derivative of N-WASP cleanly deleted for the WH1 domain (a construct that had been lacking in previous studies), but also quantitatively assessed the percentage of bound bacteria generating pedestals on transfected N-WASP-null fibroblasts. Though the WH1 domain, when combined with the VCA domain, could restore low levels of pedestal formation, the removal of the WH1 from N-WASP had no discernible effect on EPEC pedestal formation, indicating that the WH1 domain is not required. One could postulate that the low levels of EPEC pedestal formation conferred by the expression of the WH1 and VCA domains result from the additional Tir-mediated, Nck-independent actin signaling cascades, which were discovered after the initial studies of N-WASP were conducted.

Furthermore, the adaptation of the *Xenopus* oocyte extract system for use with mammalian cells provided a unique tool to rapidly evaluate the domain requirements of both Nck and N-WASP only in the context of Y474-dependent signaling. Our observation that Nck-null extracts supplemented with recombinant GST-SH3(B)-SH2 could not promote any detectable actin signaling in the absence of recombinant N-WASP gave us the opportunity to evaluate the domain requirements of N-WASP required for SH3(B)-SH2-dependent signaling. Not only did we confirm that the WH1 domain was not required for this signaling cascade, but we also determined that the addition of recombinant PRD-VCA could stimulate at least the same level of actin accumulation as full-length N-WASP, further refining the minimal requirements of N-WASP to only the PRD and VCA required for SH3(B)-SH2-mediated signaling. Consequently, the dominant EPEC-mediated actin signaling cascade appears to be far more simple than previous thought, requiring only the direct interaction between the phosphorylated Tir peptide and the SH2 domain of Nck to recruit Nck to Y474 and the direct interaction between the second SH3 domain of Nck with the PRD of N-WASP to localize the downstream actin machinery, culminating in actin assembly (Figure 1). Reconstitution of actin assembly with Tir Y474 phosphopeptide, Nck GST-SH3(B)-SH2 fusion protein, PRD-VCA and purified Arp2/3 is currently being attempted and might provide biochemical "proof" of this hypothesis.

Evaluating the Tir-mediated, Nck-independent signaling cascades

Recently, two Nck independent pathways for pedestal formation have been identified.

One utilizes Y474 while the other utilizes Y454, a tyrosine residue twenty amino acids

upstream. It is assumed that tyrosine phosphorylation is required for both of these pathways, because a double Y454F/Y474F Tir mutant is unable to generate pedestals on any cell line, but the putative (non-Nck) adaptor(s) that recognize these phosphotyrosines are unknown. Though vaccinia utilizes the eukaryotic adaptor protein, Grb2, to mediate its secondary actin signaling cascade (Scaplehorn *et al.*, 2002), Grb2 was quickly ruled out as the unknown adaptor for the Nck-independent pathway as it failed to bind to the Y454 or Y474 phosphopeptides and overexpression of the Grb2 SH2 domain failed to block pedestal formation (Campellone and Leong, 2005). Subsequently, we began an investigation of the N-WASP domain requirements for the Y474-dependent, Nck-independent signaling cascade. To facilitate this study, we generated a Nck-null, N-WASP-silenced cell line so that only the Nck-independent signaling cascades would be active upon introduction of N-WASP derivatives and challenge with EPEC. The ability of an N-WASP derivative composed of the WH1 domain and the VCA to complement this silenced cell line for Tir-mediated actin assembly indicated that in fact the original hypothesis that the WH1 domain plays a role in EPEC pedestal formation (Lommel *et al.*, 2001) may not have been in error, and that the previously observed WH1-dependent pedestals were due to the Nck-independent signaling cascades (Figure 1). Supporting this hypothesis, the efficiency of pedestal formation on the Nck-null, N-WASP silenced cells transfected with the WH1-VCA derivative is similar to that observed on Nck-proficient, N-WASP-null fibroblasts transfected with the same construct. Derivation of a clonal population of the Nck-null N-WASP silenced cells that are severely null in N-WASP would be helpful in validating this comparison.

Linking N-WASP to Tir in a Nck-independent fashion

A number of cytoskeletal and actin signaling proteins have been shown to localize to the actin pedestal and some of those factors have been reported to play a role in EPEC pedestal formation. A recent study placed dynamin, a 100kDa GTPase involved in membrane tubulation, vesicle generation and the formation of actin ruffles, at the EPEC pedestal. Localization of dynamin apparently required Nck since dynamin recruitment to adherent EPEC could only be observed in 13% of Nck-null fibroblasts as compared to 81% in Nck-proficient fibroblasts. Furthermore, the overexpression of the Nck SH2 domain, which is known to block EPEC pedestal formation, diminished the recruitment of dynamin to bound bacteria in wildtype fibroblasts (Unsworth *et al.*, 2006). However, reinterpretation of this data in the context of the now-identified Y474-dependent, Nck-independent signaling cascade could indicate that dynamin may participate in the secondary signaling cascade. Though dynamin has no domain known to be capable of binding to a phosphotyrosine, it does contain an SH3 domain that could potentially bind to the proline rich sequences in WIP, though this interaction has yet to be described. Alternatively, dynamin could simply be associating with F-actin rich structures via interactions with other actin-associated proteins such as profilin and cortactin (Schafer, 2004).

Another candidate to bridge Tir and N-WASP is cortactin, a Src-kinase family substrate capable of binding to F-actin. Composed of an N-terminal acidic domain, an alpha-helical region, a proline rich region and a C-terminal SH3 domain, cortactin has been shown to translocate to the sites of bacterial attachment (Cantarelli *et al.*, 2000). Dominant negative studies using a bank of cortactin mutants indicated that

overexpression of the proline rich region and SH3 domain of cortactin blocked EPEC pedestal formation, implicating cortactin in EPEC-mediated actin assembly. Silencing of cortactin diminished EPEC pedestal formation without compromising bacterial adhesion, and transfection of HeLa cells with a cortactin W525K point mutant that disrupts the SH3 domain resulted in significant reduction in F-actin accumulation (Cantarelli *et al.*, 2006). Most interestingly, cortactin has the ability to bind to WIP (Kinley *et al.*, 2003) which would link it to the WH1 domain of N-WASP.

However, neither dynamin nor cortactin have been shown to directly interact with Tir. Though cortactin can be immunoprecipitated from EPEC-infected HeLa cells using a Tir antibody (Cantarelli *et al.*, 2000), there is no evidence that this interaction is direct. A potential candidate to link N-WASP to Y474 is another SH3/SH2 domain containing adaptor molecule, Crk. In a comprehensive study of cytoskeletal factors localized to the EPEC pedestal, Goosney *et al* found that, Crk localizes along the length of the pedestal as well as at the tip, where actin assembly would be occurring (Goosney *et al.*, 2001). Crk plays a large role in the rearrangement of actin to facilitate the entry of another bacterial pathogen, *Shigella flexneri*, into epithelial cells. Upon invasion by *Shigella* into host cells, Crk and tyrosine phosphorylated cortactin translocate to the sites of invasion. Likewise, overexpression of both cortactin and Crk enhance bacterial uptake, thereby implicating both proteins in this cellular process (Bougneres *et al.*, 2004). Likewise, a recent study showed that the SH2 domains of Nck and CrkL both bound a specific phosphorylated tyrosine in SOCS3, a member of the suppressors of cytokine signaling family of proteins (Sitko *et al.*, 2004). Given the evidence that there is preference for binding among different SH2 domains, it is reasonable to hypothesize that the SH2

domain of CrkL could bind to phosphorylated Y474 in Tir. Additionally, a recent study of the formation of the immunological synapse, a process that requires actin rearrangement, has demonstrated the direct interaction between CrkL and WIP (Sasahara *et al.*, 2002). Consequently, CrkL may be the unknown adaptor required to link Tir to N-WASP via WIP. Many reagents are available to evaluate the role of CrkL in Tir-mediated, Nck-independent actin signaling. Silencing of CrkL in Nck-null fibroblasts may address whether this adaptor is critical to Nck-independent signaling. Likewise, pulldown or co-immunoprecipitation assays could test whether CrkL or WIP (or other actin machinery components, such as dynamin and cortactin) are integral components of the Nck-independent signaling cascades. Finally, one could test whether the addition of recombinant CrkL to Nck-null extracts boosts the level of Y474-dependent signaling to detectable levels, implying that CrkL plays an important role in this signaling cascade.

Most recently, additional roles for the kinases responsible for the critical phosphorylation events in Tir have been described. Though determining which kinases are responsible for phosphorylating Y474 has been controversial, a recent report has shown that Abl family kinases have the ability to not only phosphorylate Tir, but can also bind to the Tir Y474 phosphopeptide sequence via the kinase SH2 domain as well as interact with a polyproline region in the N-terminus of Tir via the kinase SH3 domain. It still remains unclear what the significance of this interaction is; however, disruption of the interaction between the kinase SH3 domain and the Tir polyproline domain inhibits pedestal formation as well as prevents recruitment of necessary kinases (Bommarius, *et al.*, in press). To test whether Abl kinase is responsible for mediating the interaction between Tir and N-WASP in the Y474-dependent, Nck-independent pathway, first one

must determine if the N-terminus of Tir is required for Nck-independent signaling. Transfection of a Tir derivative lacking the N-terminus into Nck-null fibroblasts and artificial clustering of Tir molecules to stimulate pedestal assembly can be used to evaluate the role of this region of Tir. However, transfection confers an increase in expression that may drive a phenotype not normally seen under native conditions. To eliminate the possibility that overexpression of Tir Δ N compensates for deficiencies, a series of Tir mutants where the polyproline region has been substituted with alanines can be used (Bommarius, *et al*, in press). Delivery of these Tir mutants via type III secretion may eliminate the possibility of a false phenotype. Additionally, co-immunoprecipitation experiments such as those previously suggested may provide insight as to whether Abl is transiently associated with the Tir-N-WASP complex or if it is responsible for linking Tir to N-WASP for the generation of Nck-independent pedestals.

Conclusions and future work

Understanding how a bacterial pathogen hijacks host cell processes not only provides insight into how the bacterium exacts damage upon its host, but also enables researchers to examine how these host cell processes occur. In the case of EPEC, initial research focused on a single tyrosine residue and how it linked the actin machinery to the host cell membrane, culminating in a complex model requiring the involvement of multiple host factors. However, the elucidation of additional signaling cascades stimulated by this pathogen required a re-evaluation of these protein requirements in a more quantitative fashion. The simplification of the Y474-mediated Nck-dependent signaling cascade has shed light on previously confusing models. The identification of the important role of the

WH1 domain for the Y474-dependent Nck-independent actin signaling pathway has resolved some of the conflicting data emphasizing the requirement of this domain for the Nck-dependent cascade while aiding in the search for the yet unknown signaling components required for actin assembly.

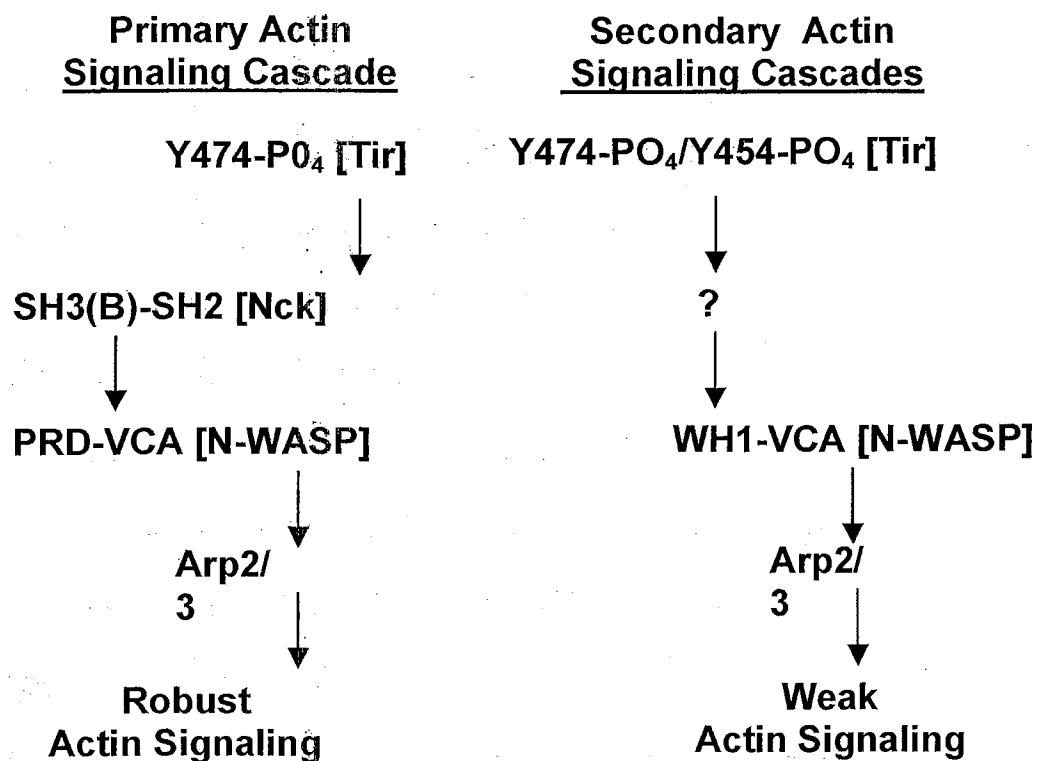


Figure 1. Simplified models of EPEC Tir-mediated actin signaling cascades

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